Study of Aminoglycoside Antibiotics Interaction with Eukaryotic Ribosomal Decoding Sites Using Bacterial Hybrid Ribosomes

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To my parents

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I. Summary

Anti-infective agents targeting the bacterial translation machinery must differentiate between prokaryotic and eukaryotic ribosomes. This discrimination provides the basis for the selectivity and toxicity of ribosomal drugs. Despite the use of ribosomal drugs for decades, we still do only in part understand the principles governing selectivity and toxicity of these agents.

Most antibiotics that bind to the ribosome have been shown to interact directly with the ribosomal RNA (rRNA). rRNA is involved in all essential steps of translation e.g. selection of cognate aminoacyl-tRNA, peptide bond formation. The site of codon-anticodon interaction is located in the small ribosomal subunit and termed “decoding region” or A (aminoacyl)-site. It is mainly composed of small-subunit rRNA, i.e., helix 44 of bacterial 16S rRNA. The decoding region is also the binding site for the 2-deoxystreptamine aminoglycosides. Recent advances in X-ray crystallography have greatly contributed to the understanding of the structural interactions between aminoglycosides and the ribosomal decoding A-site.

Aminoglycosides are polycationic amino sugars which are composed of a common core, termed neamine, in which a glycopyranosyl ring (ring I) is attached to position 4 of a 2-deoxystreptamine ring (ring II). Toxicity of 2-deoxystreptamines, i.e., ototoxicity and nephrotoxicity, limits their clinical use. The toxicity of aminoglycosides has been attributed in part to inhibition of mitochondrial protein synthesis. A single nucleotide polymorphism between bacterial 16S rRNA and its eukaryotic cytoplasmic counterpart, i.e., corresponding 16S rRNA position 1408A versus G (an adenine is found in prokaryotic ribosomes versus a guanine in eukaryotic cytoribosomes), has been suggested to form the basis for the selectivity of aminoglycoside antibiotics. The mitochondrial rRNA carries a susceptible adenine at the corresponding position providing a molecular explanation for the toxicity of these drugs. Toxicity of aminoglycosides seems also to be associated with genetic alterations of the human mitochondrial rRNA.

Investigation of rRNA structure-function relationships in most organisms is complicated by the presence of a multitude of chromosomal *rrn* genes encoding rRNA. The multiplicity of genes encoding cytoplasmic and mitochondrial rRNA in higher eukaryotes does not allow for genetic manipulation of genes encoding ribosomal nucleic acids. Likewise, most bacterial species carry multiple *rrn* operons in the chromosome. The presence of several operons hampers the isolation of *rrn* mutants due to a recessive phenotype. In addition, a mixture of wild-type and mutated ribosomes complicates biochemical investigations. These limitations in investigating structure-function relationships of bacterial ribosomal nucleic acids at a genetic level were in part met by generating *Mycobacterium smegmatis* Δ*rrnB*, the first eubacterial organism carrying a single functional *rrn* operon which is amenable to genetic manipulations. Mutant ribosomal RNA operons were introduced into *M. smegmatis* Δ*rrnB* resulting in bacterial/eukaryotic hybrid ribosomes.

1) Hybrid ribosomes carrying the rRNA decoding A-site of higher eukaryotic cytoribosomes show pronounced resistance to aminoglycoside antibiotics, equivalent to that of rabbit reticulocyte ribosomes. This finding suggests that helix 44 of the rRNA decoding A-site behaves as an autonomous domain, which can be exchanged between ribosomes of different phylogenetic domains for study of function.

2) Compared to hybrid ribosomes with the A-site of human cytosolic ribosomes, aminoglycoside susceptibility of hybrid ribosomes with the A-site of human mitochondrial ribosomes was found to be variable and to correlate with the relative cochleotoxicity of these drugs. This result provides experimental support for aminoglycoside-induced dysfunction of mitochondrial protein synthesis.

3) Recent reports on an interplay of S12 on 16S rRNA function and susceptibility to 2-deoxystreptamine aminoglycosides prompted us to study the role, if any, between *rpsL* K42R and alterations in 16S-rRNA helix 44 in more detail. We find that the non-restrictive *rpsL* K42R mutation does not affect the 2-deoxystreptamine susceptibility of various rRNA mutations in H44.

The data presented in this thesis demonstrate that the model system developed properly reflects the situation in eukaryotic ribosomes and is useful to study the interaction of aminoglycoside antibiotics with eukaryotic ribosomes. With this, we can identify residues critical for the selectivity of aminoglycosides. Ultimately, a detailed understanding of the structure-function relationships will lead to the improvement of existing antimicrobials and the rational design of effective novel compounds.

II. Zusammenfassung

Antiinfektiöse Substanzen, welche an der bakteriellen Translationsmaschinerie angreifen, müssen zwischen prokaryontischen und eukaryontischen Ribosomen unterscheiden. Eine solche Unterscheidung ist die Grundlage für die Selektivität und Toxizität von Antibiotika. Obwohl derartige Antibiotika seit Jahrzehnten eingesetzt werden, sind die Prinzipien, welche Selektivität und Toxizität dieser Substanzen bestimmen, noch immer nur teilweise verstanden.

Für die meisten Antibiotika, die am Ribosom angreifen, wurde gezeigt, dass sie direkt mit der ribosomalen RNA (rRNA) interagieren. Die rRNA ist an allen wesentlichen Schritten der Translation beteiligt, z. B. an der Auswahl passender Aminoacyl-tRNA und der Bildung von Peptidbindungen. Der Ort der Codon-Anticodon-Wechselwirkung befindet sich in der kleinen ribosomalen Untereinheit und wird als „Decodierungsregion“ oder A-Stelle (Aminoacylstelle) bezeichnet. Er besteht hauptsächlich aus rRNA der kleinen Untereinheit, d. h. Helix 44 der bakteriellen 16S-rRNA. Die Decodierungsregion ist auch die Bindestelle für 2-Desoxystreptamin-Aminoglykoside.

Aminoglykoside sind polykationische Aminozucker, die aus einem gemeinsamen Grundgerüst, dem Neamin, bestehen, in dem ein Glycopyranosylring (Ring I) an Position 4 eines 2-Desoxystreptaminrings (Ring II) gebunden ist. Die Toxizität von 2-Desoxystreptaminen, - und hier besonders die Ototoxizität -, schränkt ihre klinische Anwendung stark ein. Die Toxizität von Aminoglykosiden wird zum Teil auf eine Hemmung der mitochondrialen Proteinsynthese zurückgeführt. Hierbei wird postuliert, dass ein einzelner Nukleotidpolymorphismus zwischen der bakteriellen 16S-rRNA und ihrem eukaryontischen zytoplasmatischen Pendant an Position 1408 (ein Adenin findet man in prokaryontischen Ribosomen, ein Guanin dagegen in eukaryontischen Zytoribosomen) für die Selektivität von Aminoglykosidantibiotika verantwortlich ist. Demgegenüber enthält die mitochondriale rRNA an der entsprechenden Position ein Adenin, was eine mögliche molekulare Erklärung für die Toxizität dieser Arzneimittel liefert. Darüber hinaus scheint ein Zusammenhang zwischen der Toxizität von Aminoglykosiden und gewissen genetischen Veränderungen der humanen mitochondrialen rRNA zu bestehen.

Die Vielzahl von Genen, welche für zytoplasmatische und mitochondriale rRNA in höheren Eukaryonten kodieren, macht ihre genetische Manipulation praktisch unmöglich. Auch die meisten Bakterienarten tragen mehrere *rrn*-Operons auf dem Chromosom. Die Gegenwart mehrerer Operons erschwert die Isolation von *rrn*-Mutanten aufgrund eines rezessiven Phänotyps. Darüber hinaus erschwert eine Mischung aus Wildtyp- und mutierten Ribosomen biochemische Untersuchungen. Diese Einschränkungen bei der Untersuchung von Struktur-Funktionsbeziehungen von bakteriellen ribosomalen Nukleinsäuren auf genetischer Ebene werden durch *Mycobacterium smegmatis* Δ*rrnB* umgangen. *M. smegmatis* Δ*rrnB* ist der erste eubakterielle Organismus, der ein einziges funktionelles *rrn*-Operon trägt, das für genetische Manipulationen zugänglich ist.

1) Hybridribosomen, welche die rRNA-Decodierungsstelle (A-Stelle) der Zytoribosomen höherer Eukaryonten aufweisen, zeigen eine ausgeprägte Resistenz gegenüber Aminoglykosidantibiotika – gleichwertig mit der von Ribosomen aus Kaninchenretikulozyten. Dieses Ergebnis weist darauf hin, dass Helix 44 der rRNA-Decodierungsstelle (A-Stelle) sich als eine autonome Domäne verhält, die zur Funktionsanalyse zwischen Ribosomen verschiedenen phylogenetischen Ursprungs ausgetauscht werden kann.

2) Im Vergleich zu Hybridribosomen mit der A-Stelle humaner zytosolischer Ribosomen zeigen Hybridribosomen mit der A-Stelle humaner mitochondrialer Ribosomen unterschiedliche Aminoglykosid-Empfindlichkeit, welche mit der relativen Cochleotoxizität dieser Substanzen korreliert. Dieses Ergebnis liefert experimentelle Hinweise auf eine Aminoglykosid-induzierte Fehlfunktion der mitochondrialen Proteinsynthese.

3) Neuere Berichte über ein Zusammenspiel des ribosomalen Proteins S12 und der 16S-rRNA im Hinblick auf die Empfindlichkeit gegenüber 2-Desoxystreptamin-Aminoglykosiden veranlassten uns zu untersuchen, ob eine Funktionsbeziehung zwischen *rpsL* K42R und Veränderungen in der Helix 44 der 16S-rRNA besteht. Dabei konnten wir zeigen, dass die nichtrestriktive *rpsL*-K42R-Mutation die 2-Desoxystreptamin-Empfindlichkeit verschiedener rRNA-Mutationen in H44 nicht beeinflusst.

Die in dieser Dissertation erbrachten Daten zeigen, dass das entwickelte Modellsystem die Situation in eukaryontischen Ribosomen korrekt widerspiegelt und zur Untersuchung der Wechselwirkung von Aminoglykosidantibiotika mit eukaryontischen Ribosomen geeignet ist. Auf diese Weise können für die Selektivität von Aminoglykosiden entscheidende Strukturen identifiziert werden. Letztlich ist ein genaues Verständnis der Struktur-Funktionsbeziehungen entscheidend zur Verbesserung bestehender antimikrobieller Substanzen wie auch zur rationalen Entwicklung neuartiger Verbindungen.

1. Introduction

1.1 Mechanisms of Translation

Decades of genetic, biochemical, and biophysical characterization have established our current understanding of translation as a complex, multistep, and multicomponent process that requires intricate communication to achieve high levels of speed, accuracy, and regulation. Translation converts genetic information into proteins that execute the myriad tasks necessary for life. It is estimated that, in the simplest prokaryotic organisms, nearly half the dry weight of the cell and more than 80% of its energy are used to drive the synthesis of proteins.

Protein synthesis can be divided into four distinct phases: initiation, elongation, termination and recycling (Fig. 1). Each stage requires the coordination of multiple components of the translational machinery and the precise timing of molecular events. The role of the initiation phase is to position the ribosome correctly on the mRNA so that protein synthesis initiates at the right place in the correct reading frame. The result is a 70S ribosome programmed with the start codon of the mRNA and the initiator-tRNA located at the P-site of the ribosome, a so-called 70S initiation complex. In principle, there are three binding sites for the tRNA on the ribosome. The A-site binding aminoacylated t-RNA, the P-site holding peptidyl t-RNA, and the E-site as exit site for hydrolyzed t-RNA. The elongation phase involves the movement of tRNAs in a cyclic fashion through the three tRNA binding sites A → P → E, where the number of cycles is dictated by the length of the polypeptide being synthesized. The first step in the cycle involves binding of the aa-tRNA to the A-site, which is facilitated by a protein factor EF-Tu. EF-Tu hydrolyzes GTP and dissociates from the ribosome, allowing the A-tRNA to accommodate on the large subunit (Fig. 1). Peptide-bond formation proceeds, transferring the entire polypeptide chain from the peptidyl-tRNA in the P-site to the aminoacyl moiety of the tRNA A-site. Now the ribosome has a peptidyl-tRNA at the A-site and a deacylated-tRNA at the P-site. This ribosomal state is highly dynamic and the tRNAs move back and forth into so-called A/P (A/P denotes that the tRNA is in the A-site on the 30S and P-site on the 50S) and P/E (P/E denotes that the tRNA is in the P-site on the 30S and E-site on the 50S) hybrid states. Next, translocation of the tRNAs occurs, a process that is catalyzed by a second elongation factor, EF-G. Binding of EF-G to the ribosome locks the tRNAs in hybrid states and the subsequent translocation reaction shifts the peptidyl-tRNA from the A/P hybrid state to the P-site and the deacylated tRNA from the P/E to the E-site – the outcome being that the A-site is free to bind the next incoming aa-tRNA. When a stop signal in the mRNA enters the A-site, the ribosome is then channeled into termination and recycling phases. The stop signals are recognized by protein termination factors, RF1 and RF2, which function to hydrolyze the peptidyl-tRNA bond and release the translated polypeptide chain from the ribosome. RF1 and RF2 are recycled from the ribosome by a third release factor RF3, in a GTP-dependent fashion. The post-termination ribosome complexes are then split into subunits by the concerted action of

EF-G and the ribosome recycling factor RRF and the components recycled for the next round of translation (Fig. 1).

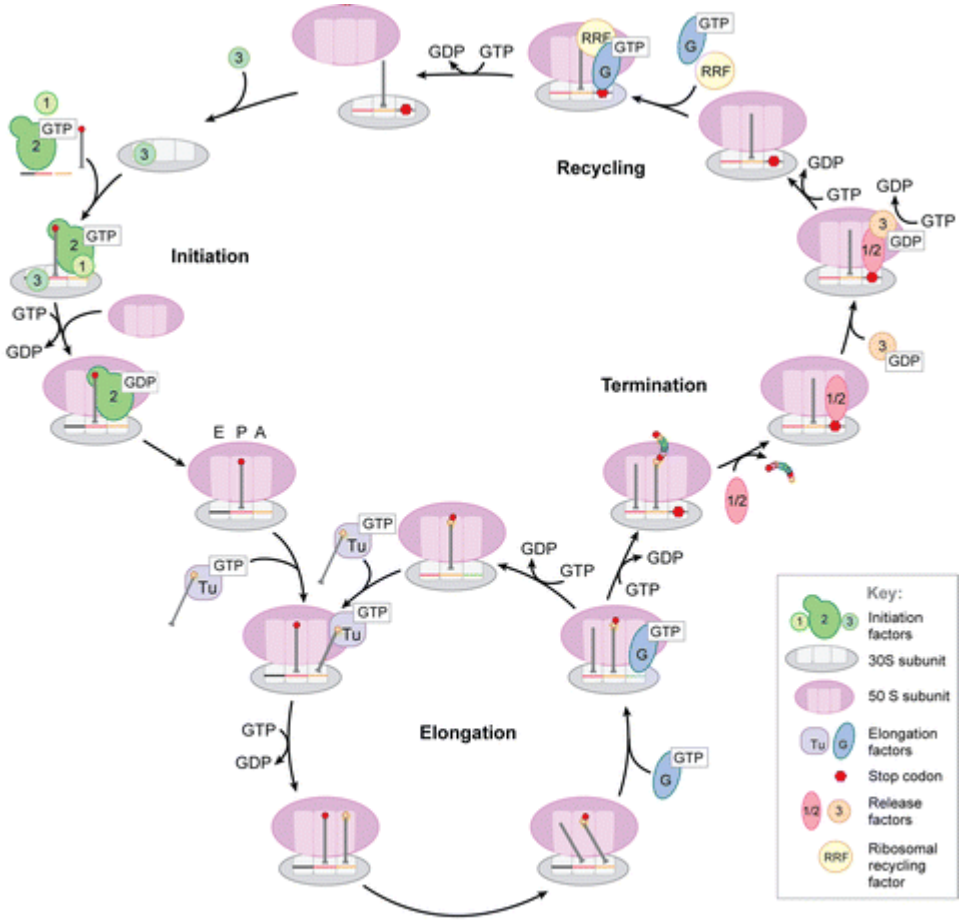


Figure 1. The prokaryotic translation cycle. Shown is the current model, which has been derived from biochemical and biophysical studies. Initiation, mediated by initiation factors 1,2, and 3 (*green-shaded circles*), culminates in the joining of 30S (*gray*) and 50S (*purple*) subunits on the mRNA message primed with initiator tRNA (*gray line* with *red circle*) in the P site. This complex, aided by the elongation factors Tu and G (*blue-shaded circles*), subsequently undergoes multiple rounds of elongation. Termination, under the control of release factors 1, 2 and 3 (*red-shaded circles*), frees the newly synthesized polypeptide upon recognition of the stop codon. Ribosomal recycling factor (*yellow circle*) and elongation factor G then prepare the translational machinery for subsequent initiation events. Abbreviations: A, ribosomal aminoacyl-tRNA site; P, ribosomal peptidyl-tRNA site; E, ribosomal exit site; G, elongation factor G; RRF, ribosome recycling factor; Tu, elongation factor Tu. Figure taken from reference (29).

1.2 Ribosome: composition and structure

The central component in translation is the ribosome, a massive (megadaltons), multisubunit biomolecular machine. The ribosome is approximately globular, its average diameter ranging from 2.5nm (*Escherichia coli*) to 2.8nm (mammalian). Mammalian ribosomes exist in two forms: cytoplasmic ribosomes and mitochondrial ribosomes (mitoribosomes). The sizes and molecular weights of ribosomes from three domains of life are listed in Table 1.

Characteristics	Bacteria	Archaea	Mitochondria	Eukarya
Ribosome size	70S	70S	55S	80S
Small subunit				
Size	30S	30S	28S	40S
Mass (MDa)	0.8	0.8	1.2	1.4
rRNAs	16S	16S	12S	18S
Number of r-proteins	20	28	33	32
Large subunit				
Size	50S	50S	39S	60S
Mass (MDa)	1.6	1.6	2.4	2.6
rRNAs	23S, 5S	23S, 5S	16S	28S, 5.8S, 5S
Number of r-proteins	34	40	52	46

Table 1: Ribosome components from the three domains of life. Table taken from reference (41).

Ribosomes are made up of two subunits, both of which consist of ribosomal RNA (rRNA) and many specific ribosomal proteins (r-proteins). Ribosomes translate genetic information stored on the messenger RNA (mRNA) into polypeptides. Figure 2A shows the crystal structure of 70S ribosome with large (50S) and small (30S) ribosomal subunits from the thermophilic bacterium *Thermus thermophilus*. The small subunit contains the decoding site (Fig. 2B) where the mRNA sequence is read in blocks of three nucleotides, called codons. Each codon denotes one of twenty different amino acids, and each amino acid is ferried to the ribosome by its own transfer RNA (tRNA) or set of tRNAs. Every tRNA has an anticodon sequence that makes a specific match with the corresponding mRNA codon. The mRNA passes through two narrow channels on the 30S subunit to be displayed at the interface decoding site, where it interacts with the tRNA anticodon (10, 33, 44, 45). The basepairing match between the tRNA anticodon and mRNA codon is checked by the decoding site within the A site of the small subunit.

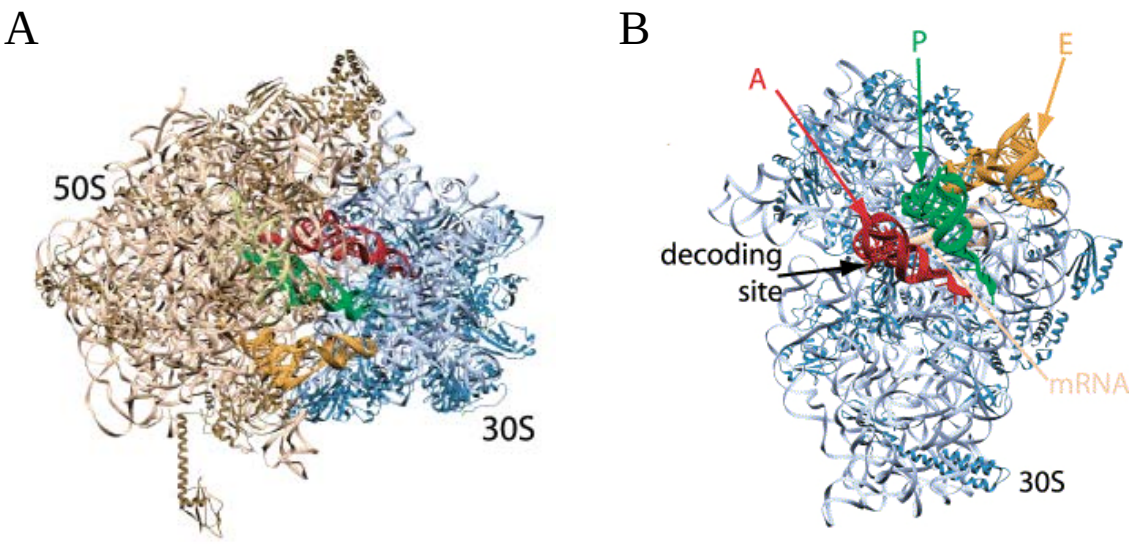


Figure 2. Crystal structure of the ribosome. (A) 70S ribosome complexed with mRNA and tRNA.(B) Exploded view of the 30S subunit in the 70S ribosome, showing the locations of A-(red), P-(green), and E-(gold) site tRNAs. Figure taken from reference (44).

1.2.1 Small subunit ribosomal proteins

The structural studies of the ribosome confirmed the predominance of rRNA at the ribosomal active sites, but also revealed that a number of r-proteins were located in positions of functional importance, for example, S12 at the decoding centre. Both proteins and rRNA are essential for optimal ribosome function. There are 21 r-proteins (S1–S21) in the small subunit of *E. coli* ribosomes and they are universally conserved through all three domains of life (Table 2).

Small Subunit			
Bacteria: *E. coli**	Archaea *H. ma**	Low Eukarya: *Yeast**	High Eukarya: *Rat**
S2	S2	S0	Sa
S3	S3	S3	S3
S4	S4	S9	S9
S5	S5	S4	S2
S7	S7	S5	S5
S8	S8	S22	S15a
S9	S9	S9	S16
S10	S10	S20	S20
S11	S11	S14	S14
S12	S12	S28	S23
S13	S13	S18	S18
S14	S14	S29	S29
S15	S15	S13	S13
S17	S17	S18	S11
S19	S19	S15	S15

Table 2: Concordance between the universally conserved ribosomal proteins in small subunit from the three kingdoms of life. Table taken from reference (58).

Binding of cognate tRNA to the A site induces a transition in the 30S subunit from the open to the closed form. This involves a rotation of the head and movement of body (see red arrows in Fig. 7A) towards the decoding site. The closed form brings elements of S12 and 16S rRNA into contact. In particular, the interactions of G530, A1492, and A1493 with the codon-anticodon helix minor groove make the transition to the closed form favorable for cognate but not near-cognate tRNA. Binding of near-cognate tRNA to the ribosome indicates that it associates weakly with the A-site codon, the 30S remains in an open conformation, similar to when the decoding centre is unoccupied.

Ribosomal protein (r-protein) S12 encoded by *rpsL* gene is a critical component of the decoding center of the 30S ribosomal subunit and involved in recognizing the codon-anticodon positions at the A-site. Mutations in the r-protein S12 are known to affect ribosomal accuracy to various extents, resulting in what is known as restrictive or non-restrictive *rpsL* mutations. Mutations in S12 that block salt-bridge formation between S12 and nucleotides of the 16S may destabilize the closed form and result in restrictive (hyperaccurate) ribosomes (9). The classical S12 mutations were isolated as streptomycin resistance mutants (5).

Crystal structures of streptomycin bound to the small ribosomal subunit of *T. thermophilus* have revealed that the lysine residue 42 (*E. coli* numbering) of ribosomal protein S12 forms contact to the phosphate backbone of 16S-rRNA helix 27 (H27) via a salt bridge to the phosphate group of residue A913. K42 also forms two hydrogen bonds with streptomycin (Fig. 3).

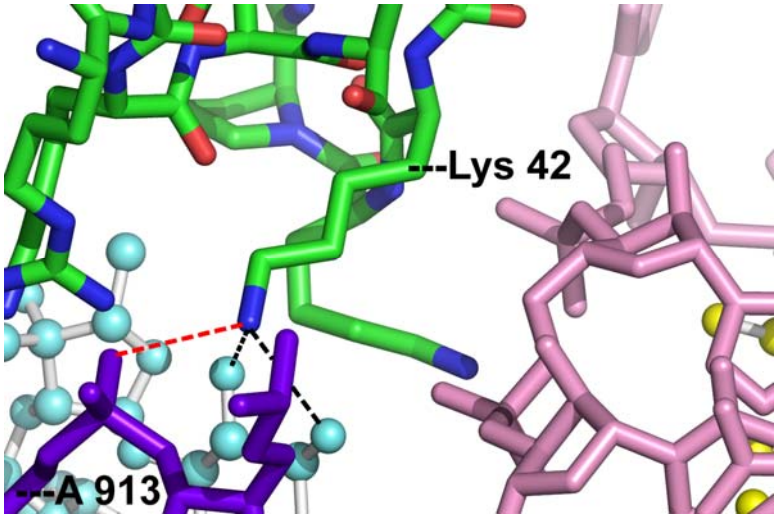


Figure 3. A model of three-dimensional crystal structure of *T. thermophilus* ribosomal decoding A-site with bound streptomycin (Protein Data Bank, 1FJG.pdb). Amino-acid residues of S12 are shown labelled according to atoms: carbon – green, nitrogen – blue, oxygen – red. H44 (pink), H27 (violet), streptomycin (light blue), Hydrogen bonds (black dotted lines) and salt bridge (red dotted line) are shown.

A number of the mutations in the r-protein S12 that confer resistance to, and in some cases even dependence on streptomycin, map within a loop of S12 that directly contacts the drug molecule (Fig. 7A) (26). Of these mutations, positions Lys42 and Lys87 directly interact with streptomycin, suggesting that the other mutations confer resistance indirectly by altering in the loop conformation.

1.2.2 Small subunit ribosomal RNA

The ribosomal RNAs (rRNAs) are at the core of the protein synthesis machinery. These RNAs were long regarded as mere scaffolds for the r-proteins but recent work has shown that the rRNAs in fact carry out the key reactions in translation. A major function of the r-proteins is ensuring the correct structure of the rRNA, allowing its tight packing around the active centres of the ribosome. In almost all organisms the small ribosomal subunit contains a single RNA species (the 18S rRNA in eukaryotes, the 12S rRNA in mitochondria and the 16S rRNA in prokaryotes). The 16S rRNA can be divided into four domains, - the decoding region is located at helix 44 (H44) of Domain IV (Fig. 4)

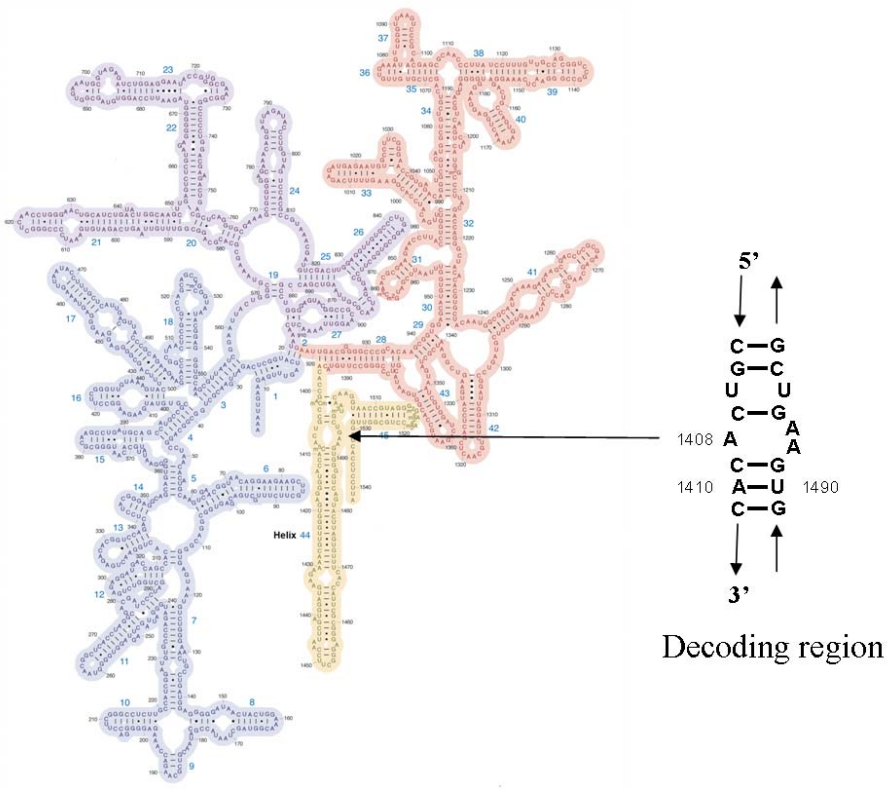


Figure 4. Secondary structure of 16S rRNA from *E. coli* showing decoding region at helix 44. Domain I (blue), Domain II (violet), Domain III (pink), and Domain IV (yellow) are shown.

1.3 Eukaryotic Ribosomes

The eukaryotic ribosomes come in two flavors: the cytoplasmic and the mitochondrial ribosomes.

1.3.1 Cytoplasm and cytoplasmic ribosomes

The cytoplasm is the part of a cell that is enclosed within the cell membrane. In eukaryotic cells, the contents of the cell nucleus are not part of the cytoplasm and are instead called the nucleoplasm. In eukaryotic cells, the cytoplasm contains organelles, such as mitochondria, which are filled with liquid that is kept separate from the rest of the cytoplasm by biological membranes. The cytoplasm is the site where most cellular activities occur, such as many metabolic pathways like glycolysis, and processes such as cell division. The inner, granular mass is called the endoplasm and the outer, clear and glassy layer is called the cell cortex or the ectoplasm.

Cytoplasmic ribosomes are present in two forms, free floating and membrane bound (bound to the rough endoplasmic reticulum). Human 80S cytoplasmic ribosomes contain 2 subunits i.e., small (40S) and large (60S). The small ribosomal subunit contains 18S rRNA and 32 proteins and the large subunit comprises the 28S, 5.8S, 5S rRNA and 46 proteins (Table 1).

1.3.2 Mitochondria and mitochondrial ribosomes

Mitochondria are found in all nucleated cells and evolved from a symbiotic relationship between aerobic bacteria and primordial eukaryotic cells (56). As the principal generators of cellular ATP by oxidative phosphorylation (OXPHOS), these double-membrane organelles provide a highly efficient route for eukaryotic cells to generate ATP from energy-rich molecules. Electrons from oxidative substrates are transferred to oxygen, via a series of redox reactions. In the process, protons are pumped from the matrix across the mitochondrial inner membrane through respiratory complexes I, III, and IV. When protons return to the mitochondrial matrix down their electrochemical gradient, ATP is synthesized via complex V (ATP synthase). Mitochondria are the only location of extra-chromosomal DNA within the eukaryotic cell (except in plant chloroplasts), and they are under the dual genetic control of both nuclear DNA and the mitochondrial genome. Although the vast majority of mitochondrial proteins (about 900) are encoded by the nuclear genome and imported into the mitochondria, mitochondria nevertheless maintain a genome that is essential for their respiratory function (56). The mitochondrial genome consists of a multicopy, circular dsDNA (mtDNA) molecule (16.6 kb in humans) that contains 37 genes. Thirteen of these genes encode protein subunits of respiratory complexes I, III, IV, and V; only complex II is solely composed of proteins encoded by nuclear genes. The mtDNA genome also encodes 22 mitochondrial tRNAs and 2 rRNAs that are essential for translation of mtDNA transcripts within the organelle.

Human 55S mitochondrial ribosomes contain 2 subunits i.e., small (28S) and large (39S). The small ribosomal subunit contains 12S rRNA and 33 proteins and the large subunit comprises the 16S rRNA and 52 proteins Table 1. Since mitochondria are believed to have arisen from endosymbiosis of a eubacterium, it had been assumed that the mitoribosome would be structurally closely related to bacterial ribosomes. However, mitochondrial ribosomes have a protein-to-RNA ratio of 69% protein:31% RNA, almost a complete reversal of the 33% protein:67% RNA in bacterial ribosomes.

1.4 Ribosome as a drug target

Protein synthesis is one of the fundamental processes in all living cells, and therefore, it is not surprising that the RNA and protein machinery of the prokaryotic ribosomes are the target of about half of the antibiotics characterized thus far (54). Among the different classes of clinically important antibiotics that interfere with protein synthesis (Fig. 5) *via* this target (*e.g.* aminoglycosides, macrolides, ketolides, lincosamides, oxazolidinones and tetracyclines), aminoglycosides (Fig. 6) represent gold standard drugs for the treatment of Gram-negative pathogens. Streptomycin, the first representative of this class of antibiotics, was discovered by Waksman *et al.* in 1944 and was the first effective antibiotic against *Mycobacterium tuberculosis*. In the following decades several milestone drugs, such as neomycin, kanamycin, tobramycin and others, were isolated from soil bacteria by intense search for natural products with antibacterial activity (52, 55, 57).

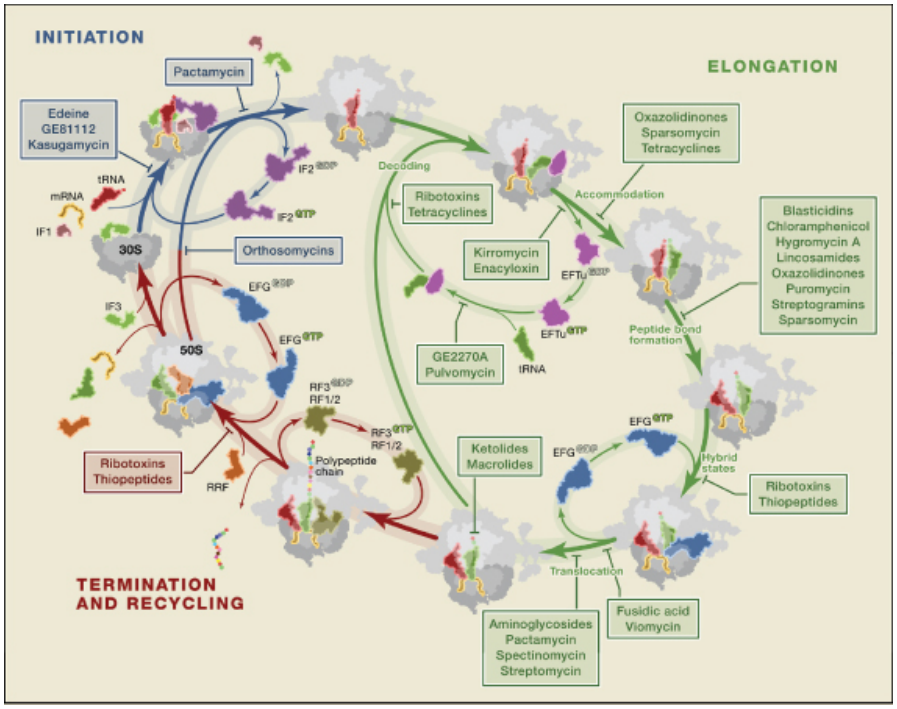
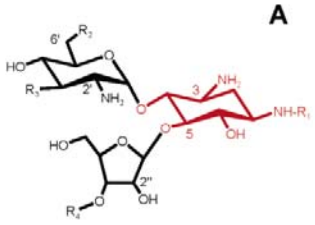


Figure 5. Sites of antibiotic action during protein synthesis. Schematic showing the sites of antibiotic action for the different stages of protein synthesis. Figure taken from reference (50).

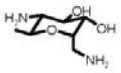
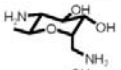
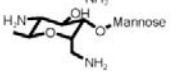
1.4.1 Aminoglycosides

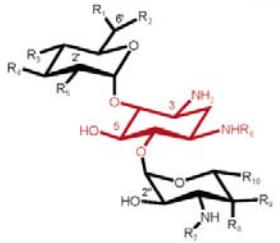
Aminoglycosides form a large family of water-soluble, polycationic amino sugars which are used as broad spectrum antibacterial agents (25, 30). Common to all aminoglycosides is the neamine core. The neamine core is composed of a sixmembered cyclitol (2-deoxystreptamine; ring II) glycosidically linked to a glucopyranosyl (ring I) (19, 39). Additional sugars are attached to position 5 or 6 of the 2-deoxystreptamine moiety to give rise to a variety of compounds categorized as 4,5- or 4,6- aminoglycosides (Fig. 6). Aminoglycosides target the ribosome by direct interaction with ribosomal RNA and they affect protein synthesis by inducing codon misreading and by inhibiting translocation of the tRNA-mRNA complex (Fig. 5) (11, 30, 33, 51)

All 2-deoxystreptamine aminoglycosides bind within an internal loop in H44 of the 30S subunit, which comprises the decoding site (Fig. 6) (35). This has been observed biochemically by chemical probing (31, 32, 60) as well as structurally in complexes of aminoglycosides bound to small RNA fragments mimicking H44 (13, 14, 27, 53) as well as to 30S subunit (9, 34, 36) or 70S ribosomes (49).

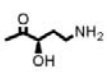
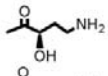
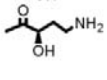


A

Antibiotic	R_1	R_2	R_3	R_4
Ribostamycin	H	NH_2	OH	H
Butirosin		NH_2	OH	H
Paromomycin	H	OH	OH	
NeomycinB	H	NH_2	OH	
Lividomycin A	H	OH	H	



B

Antibiotic	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	R_9	R_{10}
Kanamycin A	H	NH_2	OH	OH	OH	H	H	H	OH	CH_2OH
Kanamycin B	H	NH_2	OH	OH	NH_2	H	H	H	OH	CH_2OH
Tobramycin	H	NH_2	OH	H	NH_2	H	H	H	OH	CH_2OH
Dibekacin	H	NH_2	H	H	NH_2	H	H	H	OH	CH_2OH
Gentamicin B	H	NH_2	OH	OH	OH	H	CH_3	OH	CH_3	H
Gentmicin C1	CH_3	$NHCH_3$	H	H	NH_2	H	CH_3	OH	CH_3	H
Gentmicin C1A	H	NH_2	H	H	NH_2	H	CH_3	OH	CH_3	H
Gentmicin C2	CH_3	NH_2	H	H	NH_2	H	CH_3	OH	CH_3	H
Sisomicin*	H	NH_2	H	H	NH_2	H	CH_3	OH	CH_3	H
Netilimicin*	H	NH_2	H	H	NH_2	CH_2CH_3	CH_3	OH	CH_3	H
Isepamicin	H	NH_2	OH	OH	OH		CH_3	OH	CH_3	H
Arbekacin	H	NH_2	H	H	NH_2		H	H	OH	CH_2OH
Amikacin	H	NH_2	OH	OH	OH		H	H	OH	CH_2OH

*(Δ5'>4' unsaturation)

Figure 6: Chemical structures of disubstituted deoxystreptamines, the clinically most relevant class of aminoglycosides: (A) 4,5-disubstituted deoxystreptamines; (B) 4,6-disubstituted deoxystreptamines. Figure taken from reference (28).

The A site of the 30S ribosomal subunit (the decoding centre), in which the codon and anticodon pair, is made up of four different domains: the head, shoulder, platform and H44 (Fig. 7A).

A

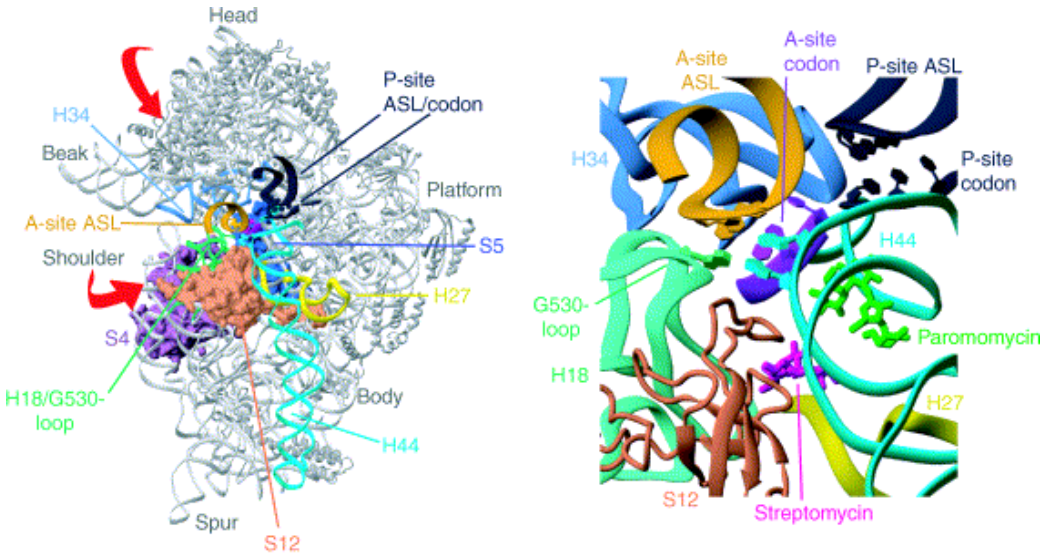


Figure 7A: The bacterial decoding center in the 30S ribosomal subunit. Overview of the 30S subunit structure, in complex with A-site tRNA anticodon stem–loop (ASL, gold). Red arrows indicate the movement of domains during the transition to the closed 30S conformation. P-site codon and tRNA-ASL (mimicked by the 3′ end of the 16S RNA and the 'spur' stem–loop of a symmetry-related molecule in the crystal) are dark grey, helices H44 cyan, and H27 yellow. In the shoulder domain, H18 with the 530-loop is turquoise, and proteins S12 (orange), S4 (violet) and S5 (dark blue, on the back of the subunit) are highlighted in space-filling representation. In the head domain, H34 is blue. Close-up of selected 30S elements around the decoding center, showing the A-site codon (purple), 16S RNA nucleotides G530 in the 530-loop (turquoise), and A1492 and 1493 in H44 (cyan) the positions of paromomycin (green) and streptomycin (pink). Remaining colors as in panel A (proteins S4 and S5 not shown) (35).

B

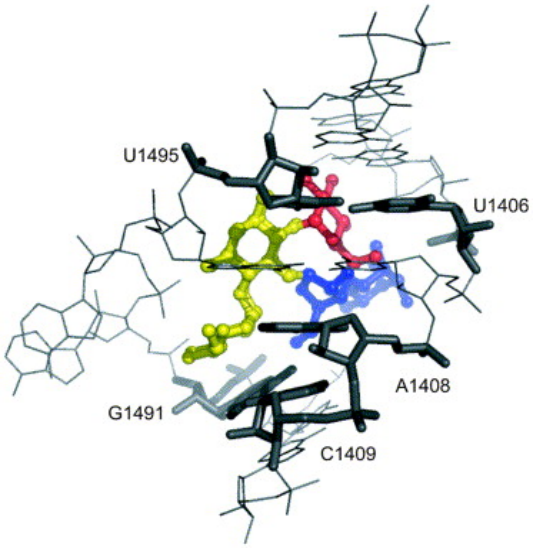


Figure 7B. The bacterial A-site (*E. coli* numbering) is a well known target for antibiotics. View of the three-dimensional structure of the A-site complexed with 4,5- and 4,6-disubstituted 2-deoxystreptamines: the common neamine core is denoted in yellow; ring III of the 4,6-compounds (tobramycin) is denoted in red; and rings III and IV of the 4,5-compounds (paromomycin) are denoted in blue. Key nucleotides of the binding site are given in bold. Figure taken from reference (7).

During the last decade, several achievements in bacterial ribosome structure determination (9) (47) along with crystal and NMR structures of bacterial A-site oligonucleotide models (14, 54, 59) have provided fascinating insights into our understanding of the decoding mechanism in prokaryotic cells and of how 2-DOS aminoglycosides induce the deleterious misreading of the genetic code. During decoding, a critical step in aminoacyl-tRNA selection is based on the formation of a minihelix between the codon of the mRNA and the anti-codon of the cognate aminoacyl-tRNA. In this process, the conformation of the A-site is changed from an "off" state, where the two conserved adenines A1492 and A1493 are folded back within the helix, to an "on" state, where A1492 and A1493 are flipped out from the A-site and interact with the cognate codon–anticodon mini-helix (34, 36). This conformational change is a molecular switch that irreversibly determines on the continuation of translation. The binding of aminoglycosides such as paromomycin to the bacterial A-site changes the conformation equilibrium of the conserved adenines A1492 and A1493 by stabilizing the "on" state conformation even in the absence of cognate tRNA–mRNA complex (Fig. 8). Thus, the affinity of the A-site for a non-cognate mRNA–tRNA complex is increased upon aminoglycoside binding, preventing the ribosome from efficiently discriminating between near-cognate and cognate complexes and leading to the assembly of proteins of incorrect sequence (37).

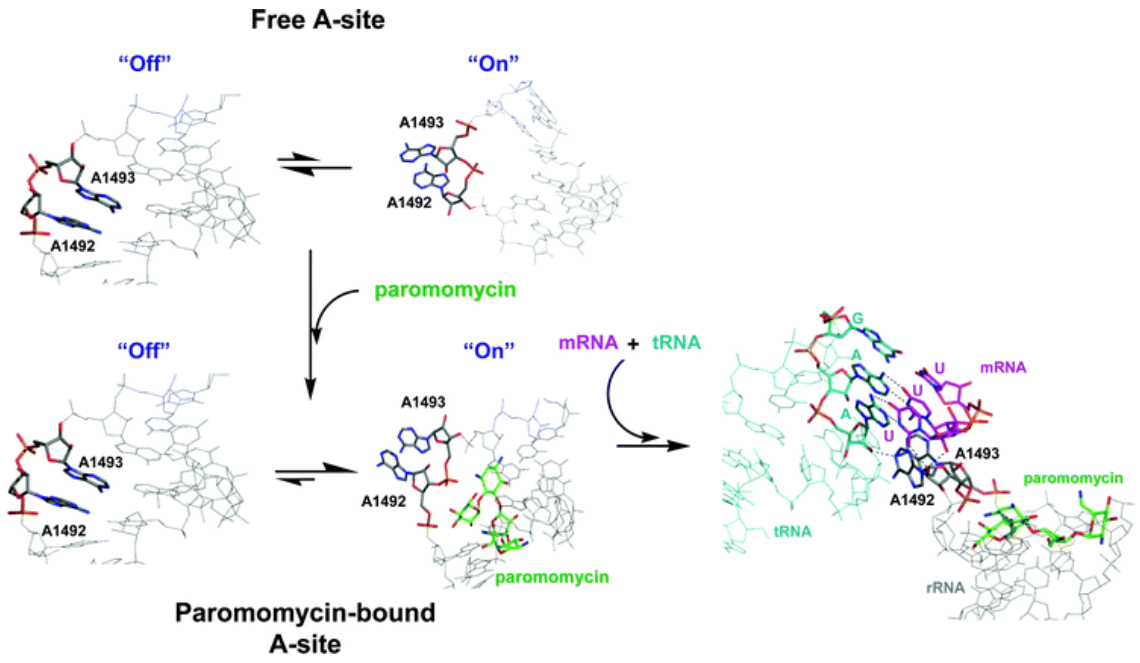


Figure 8. The molecular basis of the aminoglycoside-induced miscoding as resolved by X-ray crystal structures. At the bacterial decoding site (A-site), two flexible adenines A1492 and A1493 are in conformational equilibrium with a predominance of an intrahelical "off state" conformation. The binding of 2-DOS aminoglycoside paromomycin (green) shifts the equilibrium by stabilizing the "on state" conformation even in the absence of mRNA or tRNA. In the "on state" conformation the A1492 and A1493 are able to create hydrogen bonds with the bases of the mini-helix formed by the near-cognate tRNA anticodon (cyan) and the mRNA codon (magenta) leading to miscoding (16).

1.4.2 Selectivity and toxicity of Aminoglycosides

Drugs targeting the ribosome are characterized by two features: specificity and toxicity (6, 23, 24, 38, 40). During the past decades, considerable evidence has accumulated demonstrating that the nucleic acid component of the ribosome is key to binding many of the ribosomal drugs, rather than the numerous ribosomal proteins (15, 31, 33). Recent data from X-ray crystallography have not only confirmed this suggestion but also provided details of drug-target interactions at the atomic level by revealing how antibiotic binding occurs (4, 17, 48, 59, 61).

The components of the cytoribosome are encoded by chromosomal genes as are the mitoribosomal proteins. However, the rRNA components of the mitoribosome are encoded by the mitochondrial genome. Although the basis for drug related toxicity of the ribosomal inhibitors is unknown, several lines of evidence point to mitoribosomes as the Achilles heel of ribosomal antibiotics, because: (i) mitochondrial ribosomes are more related to the prokaryotic ribosome than to the eukaryotic cytoplasmic ribosome; (ii) toxicity *in-vivo* correlates with activity *in-vitro*, i.e., those antibiotics which exhibit in vitro activity on mitoribosomes are associated with toxicity in vivo; (iii) familial hypersensitivity to aminoglycosides (drug-induced deafness) is associated with specific mutations in mitochondrial rRNA (43).

It has been suggested that the analysis of drug resistance mutations in bacteria allows one to understand the basis of specificity for drugs targeting the ribosome (8). Central to this hypothesis is the concept of ‘informative sequence positions’ _ i.e., the identification of polymorphic nucleotides as a determinant of drug resistance in bacteria. The identification of a polymorphic residue as determinant of ribosomal resistance provides information about the selectivity of a ribosomal antibiotic, i.e., whether a drug affects the prokaryotic as opposed to the eukaryotic ribosome. The basis for this hypothesis was initially established by investigating bacterial alterations within the ribosome mediating resistance to aminoglycosides. The conclusion from these studies was that the selectivity of these agents is largely due to a single nucleotide position within the rRNA, e.g., the identity of the base at 16S rRNA position 1408. According to this hypothesis, selectivity of the aminoglycosides is due to the natural insensitivity of eukaryotic cytoplasmic ribosomes conferred by a guanine at 16S rRNA position 1408; conversely the toxicity of aminoglycosides (at least irreversible ototoxicity) is due to the natural susceptibility of mitoribosomes, which carry a susceptible bacterial adenine at this sequence position (Fig. 9) (8).

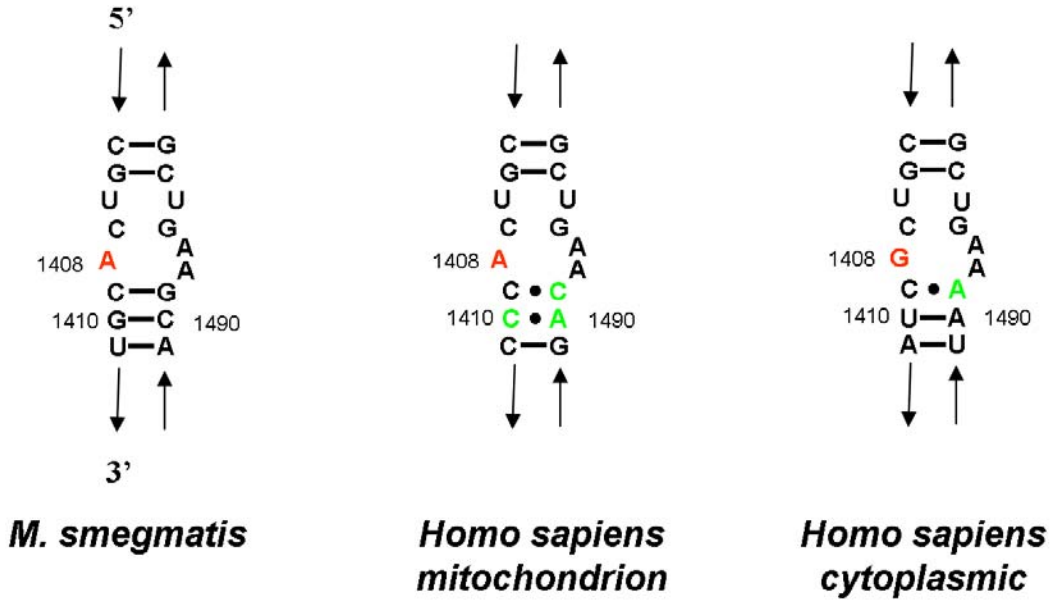


Figure 9. 16S rRNA decoding region: secondary structures.

1.5 Ribosomal dysfunction in Mitochondria

Mitochondria are vital components of all nucleated cells. Mitochondrial diseases are a clinically heterogeneous group of disorders that arise as a result of dysfunction of the mitochondrial respiratory chain. They can be caused by mutations of nuclear DNA or mitochondrial DNA (mtDNA). Nuclear gene defects may be inherited in an autosomal recessive manner or an autosomal dominant manner. Mitochondrial DNA defects are transmitted by maternal inheritance. A male does not transmit the mtDNA mutation to his offspring. Some mitochondrial disorders only affect a single organ (such as the eye in Leber hereditary optic neuropathy [LHON]), but many involve multiple organ systems and often present with prominent neurologic and myopathic features. Mitochondrial dysfunction has pleiotropic effects in multicellular organisms. Mitochondrial disorders may present at any age. In general terms, nuclear DNA mutations present in childhood and mtDNA mutations (primary or secondary to a nuclear DNA abnormality) present in late childhood or adult life. Common clinical features of mitochondrial disease include ptosis, external ophthalmoplegia, proximal myopathy and exercise intolerance, cardiomyopathy, sensorineural deafness, optic atrophy, pigmentary retinopathy, and diabetes mellitus.

Mutations in the decoding A-site of mitochondrial 12S rRNA have been associated with deafness (2, 3). In particular, the single-nucleotide alterations A1555G and C1494U have been identified as a major source of nonsyndromic deafness (43, 62). By themselves, these mutations produce a clinical phenotype that may range from severe congenital deafness through moderate progressive hearing loss of later onset to normal hearing (12). Interestingly, and in addition to the genetic predisposition, mutations A1555G and C1494U render affected individuals hypersusceptible to aminoglycoside autotoxicity.

1.6 Model system

Investigations on structure-function relationships of mitochondrial rRNA in higher and lower eukaryotes are mainly hampered by the lack of experimental genetic models (18, 19, 21, 22). Genetic manipulation of the rRNA component of eukaryotic ribosomes has proven exceedingly difficult due to the high copy number of corresponding operons. Higher eukaryotes have so far resisted any genetic manipulation of their ribosomal nucleic acids, virtually abolishing the possibility to test hypotheses by experimentation. Even most eubacteria harbor multiple rRNA operons (e.g. *E. coli*: 7; *Bacillus subtilis*: 10; *Streptomyces ambofaciens*: 4) making genetic studies of rRNA difficult. In the mid 90s, genetic procedures were developed which permitted the construction of eubacteria carrying a single functional rRNA operon (46) (1). These single rRNA allelic microorganisms allow mutagenesis of their ribosomal nucleic acids to result in cells containing homogeneous populations of mutant ribosomes (42).

In the present thesis, and with the view to study the interaction of aminoglycoside antibiotics with eukaryotic ribosomes, we replaced a central 34-nucleotide part of the bacterial drug binding pocket in 16S rRNA H44 of *Mycobacterium smegmatis* with its eukaryotic counterpart, resulting in bacterial hybrid ribosomes with a fully functional eukaryotic rRNA decoding site. For this, the recently described *M. smegmatis* mc^2 155 Δ*rrnB* (20) was used for all genetic manipulations. Using a strategy of unmarked deletion of chromosomal rRNA operons combined with RecA mediated site-directed mutagenesis, the decoding region was engineered.

For functional characterization of these hybrid ribosomes and to study a possible cause-effect relationship of disease-associated mitochondrial rRNA mutations, we assessed drug susceptibility *in-vivo*, by minimal inhibitory concentration assays. We also studied translational activity, drug susceptibility, drug induced misreading, and decoding accuracy *in-vitro* by cell free translation assays.

The model system was used to:

i) test the effect of ribosomal antibiotics on translation.
ii) identify mode of action of drugs and their interaction with ribosomes.
iii) study structure-function relationships within a defined region of rRNA.
iv) investigate the influence of rRNA mutations on protein synthesis.
v) investigate disease associated alterations in the small subunit rRNA.
vi) determine residues critical for selectivity and specificity of aminoglycosides.
vii) compare the functionality of humanized hybrid ribosomes to that of eukaryotic cytoplasmic ribosomes.
viii) study the functionality of hybrid protozoan parasitic ribosomes

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Engineering the rRNA decoding site of eukaryotic cytosolic ribosomes in bacteria

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ABSTRACT

Structural and genetic studies on prokaryotic ribosomes have provided important insights into fundamental aspects of protein synthesis and translational control and its interaction with ribosomal drugs. Comparable mechanistic studies in eukaryotes are mainly hampered by the absence of both high-resolution crystal structures and efficient genetic models. To study the interaction of aminoglycoside antibiotics with selected eukaryotic ribosomes, we replaced the bacterial drug binding site in 16S rRNA with its eukaryotic counterpart, resulting in bacterial hybrid ribosomes with a fully functional eukaryotic rRNA decoding site. Cell-free translation assays demonstrated that hybrid ribosomes carrying the rRNA decoding site of higher eukaryotes show pronounced resistance to aminoglycoside antibiotics, equivalent to that of rabbit reticulocyte ribosomes, while the decoding sites of parasitic protozoa show distinctive drug susceptibility. Our findings suggest that phylogenetically variable components of the ribosome, other than the rRNA-binding site, do not affect aminoglycoside susceptibility of the protein-synthesis machinery. The activities of the hybrid ribosomes indicate that helix 44 of the rRNA decoding site behaves as an autonomous domain, which can be exchanged between ribosomes of different phylogenetic domains for study of function.

INTRODUCTION

Accurate decoding of genetic information is a crucial step in protein synthesis. Genetic, biochemical and structural data provide evidence for a functional role of ribosomal RNA in mRNA decoding and tRNA selection (1–3). The functional relevance of rRNA residues in codon–anticodon recognition and stabilization is reflected in their universal conservation throughout the three phylogenetic domains of life. While this holds true for most nucleotides in helices 18, 34 and 44 of the small subunit rRNA that form the aminoacyl-tRNA acceptor site (A site), critical variations have evolved between different phylogenetic domains, most prominent in helix 44, the penultimate stem of 16S rRNA (4). These variations most likely account for a decoding region that has been highly optimized in the context of evolutionary differentiation.

Aminoglycosides are a class of structurally related antibiotics which interfere with decoding by binding to the A site of small subunit rRNA (5). These antibiotics preferentially target prokaryotic over eukaryotic ribosomes and affect protein synthesis by inducing codon misreading and inhibiting tRNA translocation (6–8). The binding site is located within a conserved loop of helix 44, which in part shows phylogenetic sequence variability, e.g. at position 1408 and at base pair 1409–1491 (9) (rRNA residues are numbered according to *Escherichia coli* nomenclature; see also Figure 2). Crystal structures of various bacterial 30S ribosomal particles in complex with different ligands, e.g. tRNA and antibiotics, have revealed the molecular mechanisms of the decoding step at atomic resolution (10,11). In the absence of high-resolution X-ray crystal structures of eukaryotic ribosomes, model oligonucleotides mimicking the ribosomal decoding site have been used for structural analysis of the A site in human cytosolic ribosomes (12).

Site-directed mutagenesis has been used in prokaryotes to study the functional relevance of individual drug–nucleotide contacts in aminoglycoside–ribosome interaction (13). A detailed analysis of the aminoglycoside target site in ribosomes of higher and lower eukaryotes is complicated by the complexity of eukaryotic rRNA genetics. Genetic manipulation of the rRNA component of eukaryotic ribosomes has proven exceedingly difficult

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The authors wish it to be known that, in their opinion, the first three authors should be regarded as joint First Authors.

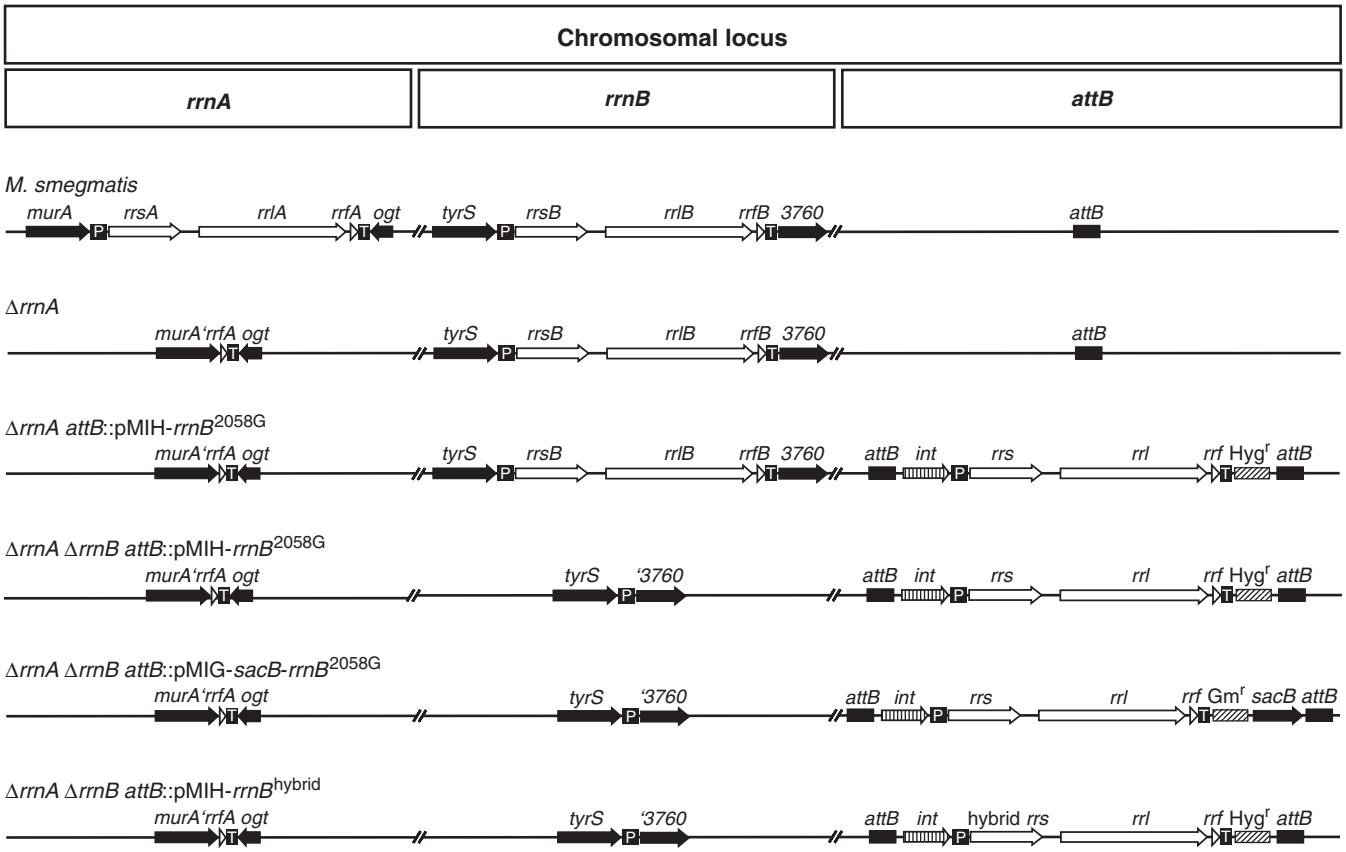

Figure 1. Sequential strategy for the generation of a plasmid-rRNA exchange system. From top: Following deletion of chromosomal *rrnA*, a complementation vector pMIH-*rrnB*^{2058G} carrying a functional *rrn* operon was introduced to the chromosomal *attB* site. Subsequent deletion of *rrnB* resulted in *M. smegmatis* Δ*rrnA* Δ*rrnB attB*::pMIH-*rrnB*^{2058G}, in which ribosomal RNA is exclusively transcribed from the plasmid. From there, a plasmid-rRNA exchange system was established by replacing pMIH-*rrnB*^{2058G} with pMIG-*rrnB-sacB*. Transformation with hybrid rRNA genes pMIH-*rrnB*^{hybrid} and selection on sucrose resulted in *M. smegmatis* Δ*rrnA* Δ*rrnB attB*::pMIH-*rrnB*^{hybrid} with homogenous populations of hybrid ribosomes.

due to the problem of the high copy number of corresponding operons. Higher eukaryotes have so far resisted any genetic manipulation of their ribosomal nucleic acids, virtually abolishing the possibility to test hypotheses by experimentation. Even in lower eukaryotic organisms, such as yeast, the presence of 100–200 tandemly repeated copies at the *RDN* locus, has made genetic studies of rRNA difficult (14–16).

The eukaryotic ribosome differs significantly from the prokaryotic one (17). It is impossible to predict the contribution of additional, phylogenetically diverse ribosomal elements and characteristics, as in the eukaryotic ribosome, to aminoglycoside susceptibility. It is, for instance, commonly assumed that translation in mammalian cells functions with higher fidelity than that of bacteria (18). To address this question experimentally, we have performed domain shuffling experiments in rRNA. Using genetic techniques, we successfully engineered a functionally critical domain in the ribosome by replacing the bacterial A-site rRNA of helix 44 with its counterpart from higher and lower eukaryotes. For functional characterization of these hybrid ribosomes, we studied their susceptibility to antibiotics targeting the ribosomal A site and which are known to affect bacterial versus eukaryotic ribosomes to different extents.

MATERIALS AND METHODS

Construction of the *rrn* plasmid exchange system

Starting with *Mycobacterium smegmatis* mc^2-155 SMR5 (19), a single-rRNA allelic strain bearing a deletion of ~5 kb in the chromosomal *rrnA* operon was constructed by gene replacement. The deletion strategy for chromosomal rRNA operons is shown in Supplementary Figure 1. Subsequently and following integration of the *rrnB*-replacement vector into the chromosomal *rrnB* locus, the strain was transformed with integration-proficient vector pMIH-*rrnB*^{2058G} for rRNA complementation. pMIH-*rrnB*^{2058G} is a derivative of plasmid pMV361-*rrnB*^{2058G} (20), in which the kanamycin resistance cassette has been replaced with a hygromycin resistance cassette. Counterselection and genetic screening for deletion of the chromosomal *rrnB* locus resulted in strain *M. smegmatis* Δ*rrn* pMIH-*rrnB*^{2058G} bearing unmarked full deletions of the chromosomal *rrnA* and *rrnB* loci. In this strain, ribosomal RNA is exclusively transcribed from the complementation vector (Figure. 1).

To establish a genetic system for efficient plasmid exchange, an integration-proficient complementation vector pMIG-*rrnB-sacB* was designed that carries the *rrnB* wild-type operon, a gentamicin resistance cassette,

and the sucrose sensitivity marker *sacB*. This vector was used to replace pMIH-*rrnB*^{2058G} in strain *M. smegmatis* Δ*rrn* pMIH-*rrnB*^{2058G}, resulting in strain *M. smegmatis* Δ*rrn* pMIG-*rrnB-sacB*. The latter was used as plasmid exchange system in which the *rrnB* wild-type operon can be efficiently replaced with an *rrnB* mutant operon by transformation and selection on sucrose-containing media (Figure. 1).

Construction of mutant strains with hybrid ribosomes

Site-directed mutagenesis of rRNA genes was performed by PCR mutagenesis as described previously (21). In brief, hybrid rDNA oligonucleotides comprising the eukaryotic helix 44 decoding site sequence were used for gene amplification. Using restriction endonuclease recognition sites present in the operon, rRNA gene fragments in plasmid pMIH-*rrnB* were replaced to construct pMIH-*rrnB*^{hybrid}, the plasmids coding for hybrid 16S rRNA genes. *Mycobacterium smegmatis* Δ*rrn* pMIG-*rrnB-sacB* was transformed with the various pMIH-*rrnB*^{hybrid} constructs and selected on hygromycin plates containing sucrose (Figure 1). Successful plasmid exchange was controlled by sequence analysis.

Minimal inhibitory concentrations

Broth microdilution tests were performed in a microtiter plate format as described previously (22). In brief, bacterial strains were cultured on Luria–Bertani (LB) agar plates at 37°C. Freshly grown cultures were resuspended in LB broth supplemented with 0.05% of Tween 80, diluted to an absorbance at 600 nm of 0.025, and incubated in the presence of 2-fold serial dilutions of the following 2-deoxystreptamine aminoglycosides: paromomycin, neomycin, geneticin (G418), gentamicin, netilmicin, tobramycin and kanamycin A (Sigma). After incubation at 37°C for 72 h, the minimal inhibitory concentration (MIC) was recorded as the lowest concentration of drug inhibiting visible growth.

Ribosome purification

Approximately 8 g of wet bacterial cell mass were resuspended in 25 ml of homogenization buffer (HB; 20 mM Tris–HCl pH 7.4, 100 mM NH_4Cl, 10.5 mM $MgCl_2$, 0.5 mM EDTA and 3 mM 2-mercaptoethanol) and lyzed with a French Pressure Cell (American Instrument Company, Maryland) at 16 000 psi, as described previously (23). The lysate was supplemented with DNase (RQ1 RNase-free, Promega, 2 U per gram cell mass), incubated on ice for 10 min, and adjusted with HB to a volume of 70 ml. Pre-cooled alumina (1.5 g per gram cell mass) was added to the cell lysate, stirred on ice for 20 min, and removed by low-speed centrifugation (10 min at 2000*g*; all centrifugations were performed at 4°C). Cell debris was sedimented by centrifugation for 30 min at 10 000*g* and the lysate was passed through a tea filter. Centrifugation of the lysate for 60 min at 32 500*g* resulted in the S-30 supernatant. Sixteen milliliter portions of the supernatant were layered on 9 ml sucrose cushions (SC; 20 mM Tris–HCl pH 7.4, 350 mM NH_4Cl, 10.5 mM $MgCl_2$, 0.5 mM EDTA, 1.1 M sucrose and 3 mM 2-mercaptoethanol) and centrifuged for 15 h at 110 000*g* (Beckman Coulter Optima™ L-80 XP Ultracentrifuge) to separate ribosomal particles from the S-100 supernatant. The S-100 fraction was dialyzed for 24 h (6000–8000 MWCO, Spectra/Por, Serva) with three intermittent changes of buffer A (50 mM Tris–HCl pH 7.5, 70 mM NH_4Cl, 30 mM KCl and 7 mM $MgCl_2$), concentrated (Viva spin, 10 000 MWCO, Viva Science), and stored at −80°C. Ribosome pellets from the S-100 preparation were resuspended in washing buffer (WB; 20 mM Tris–HCl pH 7.4, 350 mM NH_4Cl, 10.5 mM $MgCl_2$, 0.5 mM EDTA and 7 mM 2-mercaptoethanol) and passed twice through sucrose cushions (6 h at 180 000*g* and 16 h at 83 000*g*). The final ribosome pellets were resuspended in buffer A, incubated for 30 min at 4°C, dispensed into aliquots, and stored at −80 C after shock freezing in liquid nitrogen. 70S ribosome concentrations were determined by absorption measurements on the basis of 23 pmol per A_{260} unit.

Qualitative evaluation of ribosome preparations

Integrity of 70S ribosomes was determined by analytical ultracentrifugation (14 h at 70 000*g*) through a 10–40% sucrose gradient in both association buffer (buffer A) and dissociation buffer (50 mM Tris–HCl pH 7.5, 70 mM NH_4Cl and 30 mM KCl). 70S, 50S and 30S ribosome fractions in different gradient layers were detected and quantified by absorption at 254 nm. Functional activity of purified ribosomes was determined by assessing their capacity to form initiation complexes. 70S ribosomes (0.5 μM) were incubated in buffer A with m022 SD-MFTI-mRNA at 1.5 μM (5′-GGCAAGGAGGUAAAUAAUG UUCACGAUC-3′; obtained from Dharmacon), initiation factors (IF) 1 and 3 from *M. smegmatis*, IF-2 from *E. coli* (1 μM each), GTP (1 mM), and [^{3}H]-fMet-tRNA^{fMet} (1 μM) for 60 min at 37°C; purified tRNA and IF2 from *E. coli* were a kind gift from Marina Rodnina. The initiation complexes formed were bound to nitrocellulose filters (Sartorius, pore size 0.45 μm), washed with 15 ml cold buffer A, dissolved in 10 ml scintillation cocktail (Filtersafe, Zinsser Analytic) and quantified in a Liquid Scintillation Analyzer (Tri-Carb 2900 TR, PerkinElmer).

Cell-free $(UUU)_{12}$ translation assays

Cell-free translation reactions in buffer A (pH 7.5) were prepared on ice and contained *M. smegmatis* tRNA^{bulk} (0.5 mg/ml), amino acids mixture (30 μM each) lacking phenylalanine and/or leucine, 10% (v/v) S100 extract, energy mix [DTT (1 mM), GTP (1 mM), ATP (4 mM), PEP (5 mM)], pyruvate kinase (0.1 mg/ml), and polyamines [spermidine (2 mM) and putrescine (8 mM)]. The reaction mixture was preincubated with radiolabeled phenylalanine and/or leucine (30 μM; obtained from Amersham) at 37°C for 15 min. The translation reaction was started by addition of ribosomes to a final concentration of 0.25 μM and $(UUU)_{12}$-mRNA (5′-GCGGCAA GGAGGUAAAUA AUG $(UUU)_{12}$ UAA GCAGG-3′, obtained from Dharmacon) to 1 μM; serially diluted aminoglycoside antibiotics were added simultaneously. Following incubation at 37°C for 60 min, the reaction was

stopped by addition of KOH to 0.5 M and subsequent hydrolysis at 37°C for 30 min. Synthesized polypeptides were precipitated with 200 µl of 5% tri-chloro-acetic acid (TCA) for 10 min on ice and bound to nitrocellulose filters (Sartorius, pore size 0.45 µm). Filter-bound polypeptides were washed with cold 30% 2-propanol and quantified in 10 ml of scintillation cocktail. Data analysis was performed with Prism (GraphPad Software Inc.). Best-fit nonlinear regression was used to define the 100% value of polypeptide synthesis and to calculate the aminoglycoside IC_{50} values of dose-dependent inhibition (Figure 3 and Table 2).

Cell-free luciferase translation assays

Purified 70S hybrid ribosomes were used in a coupled transcription–translation reaction of firefly luciferase (plasmid pBESTluc, Promega). A typical reaction (15 µl volume) contained 0.25 µM 70S ribosomes, 300 ng DNA, 40% (v/v) of *M. smegmatis* S100 extract, 100 µM amino acid mixture, and RNasin (40 U, Promega). rNTPs, tRNAs and energy were supplied by addition of commercial S30 Premix Without Amino Acids (Promega). Serially diluted aminoglycosides were added and the reaction mixture was incubated at 37°C for 60 min. The reaction was stopped on ice, 100 µl of luciferase assay substrate (Promega) was added, and the bioluminescence was measured in a luminometer (Bio-Tek instruments, FLx800). Data analysis was performed with Prism (GraphPad Software Inc.). Best-fit nonlinear regression was used to define the 100% value of luciferase synthesis and to calculate the aminoglycoside IC50 values of dose-dependent inhibition (Figure 4 and Table 3).

Rabbit reticulocyte translation assays

Cytosolic ribosomes present in commercially available rabbit reticulocyte lysate (Promega) were used for *in vitro* translation of a firefly luciferase control mRNA (Promega). A standard 30 µl reaction contained 20 µl reticulocyte lysate, 500 ng of luciferase mRNA, an amino acid mixture (100 µM each), and RNasin. Serially diluted aminoglycosides were added and the reaction mixture was incubated at 30°C for 90 min. After incubation, 100 µl of luciferase assay substrate (Promega) was added and luciferase activity was determined.

RESULTS AND DISCUSSION

The two chromosomal *rrn* operons in *M. smegmatis* were inactivated to produce a strain in which ribosomal RNA is exclusively transcribed from plasmid-encoded rRNA genes. Operon inactivation was achieved by means of unmarked deletion mutagenesis spanning the complete rRNA gene sequences and part of the promoter region (Supplementary Figure 1). For complementation, a fully functional wild-type *rrnB* operon encompassing promoter and termination sequences was cloned into the integration-proficient plasmid pMIG-*sacB*. In *M. smegmatis* Δ*rrn* pMIG-*sacB*-*rrnB*, the wild-type rRNA operon carried on pMIG-*sacB* is efficiently replaced with a mutant rRNA operon by plasmid exchange following transformation with integration-proficient plasmid pMIH-$rrnB^{mut}$ (for details, see the Materials and Methods section and Figure 1). The rRNA plasmid exchange system was used to replace the bacterial 16S rRNA helix 44 within the decoding region with its homologous structures from parasitic kinetoplastid protozoa. The kinetoplastid genera *Leishmania* and *Trypanosoma* comprise species that are responsible for leishmaniasis and trypanosomiasis, diseases that are widespread in many tropical and subtropical countries. The insect trypanosome *Blastocrithidia* is characterized by an rRNA decoding site with an adenine at the position homologous to 16S rRNA residue 1408, and therefore represents a very rare trait within the eukaryotic domain. We also constructed human–bacterial hybrid ribosomes by introducing the human helix 44 rRNA homolog into functional ribosomes of strain *M. smegmatis* Δ*rrnB* by a method described previously (13). Both approaches resulted in bacterial mutant strains with homogenous populations of hybrid ribosomes in which a 34 nucleotide portion of helix 44 was substituted with its counterpart of eukaryotic small-subunit rRNA (Figure 2). Together with A-site residues in helices 18, 34 and 44 that are universally conserved throughout the phylogenetic domains, the engineered decoding site resembles that of eukaryotic cytosolic ribosomes. Engineering the eukaryotic decoding sites into bacterial ribosomes had relatively little effect on cell growth, with only slightly increased generation times as compared to wild-type cells (generation times: wild-type 3.7 ± 0.3 h; *Leishmania* hybrids 4.8 ± 0.5 h; *Blastocrithidia* hybrids 4.2 ± 0.1 h; *H. sapiens* hybrids 5.1 ± 0.6 h).

A bacterial oligoribonucleotide analog of 16S rRNA helix 44 has been shown to interact with both antibiotic and RNA ligands of the 30S subunit in a manner that correlates with normal subunit function (24). Since the decoding-site rRNA in the hybrid ribosomes is identical to that of eukaryotic ribosomes, the hybrid ribosomes may serve as a model to investigate specific features associated with the eukaryotic A-site rRNA structure. Towards this end, we investigated the susceptibility of the hybrid ribosomes to the 2-deoxystreptamine antibiotics paromomycin, neomycin, geneticin (G418), gentamicin, netilmicin, tobramycin and kanamycin A (see Supplementary Figure 2 for chemical structures). In MIC assays, which determine growth inhibition at the whole cell level, *M. smegmatis* mutants carrying human–bacterial hybrid ribosomes were highly resistant to all aminoglycoside antibiotics tested except for geneticin (Table 1). Relative resistance of the human hybrids to geneticin, calculated as the ratio of mutant to wild-type MIC, was 8 to 16 as compared to ≥ 1024 for paromomycin, neomycin, gentamicin, netilmicin, tobramycin, and kanamycin. The *Leishmania* hybrids showed high-level resistance to neomycin, gentamicin, netilmicin, tobramycin and kanamycin, with relative resistance levels of ≥ 1024. However, the *Leishmania* hybrids showed distinct susceptibility to paromomycin and geneticin, i.e. aminoglycosides with a hydroxyl group at position 6′ of ring I. *Mycobacterium smegmatis* hybrids with a *Blastocrithidia* decoding region were susceptible to all aminoglycoside antibiotics; the minimal inhibitory concentrations of the 4,6- and

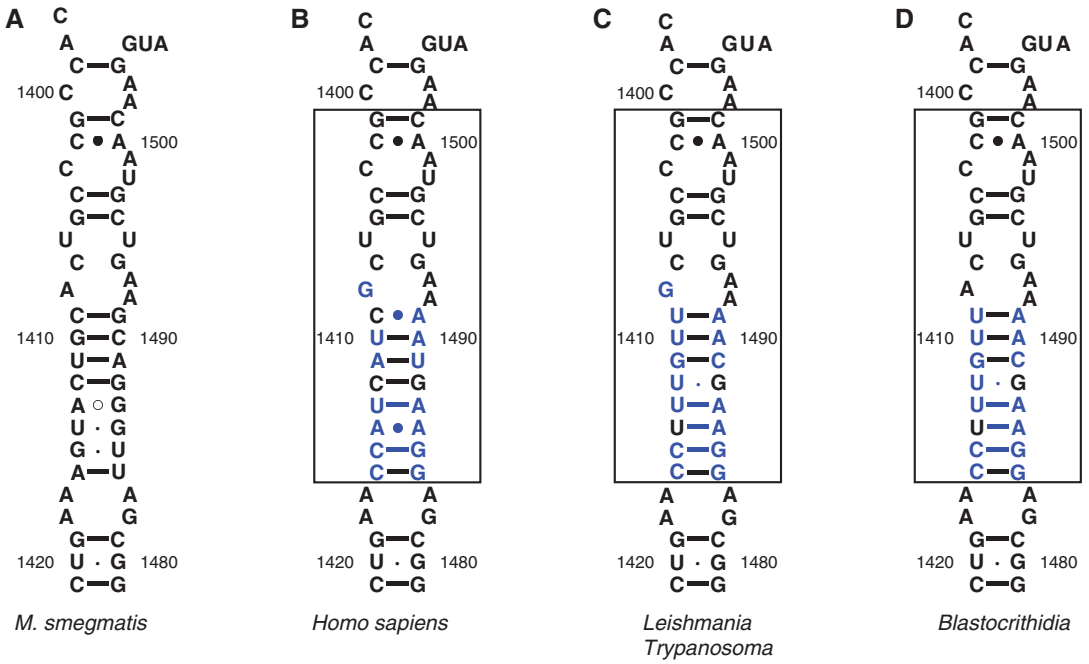

Figure 2. 16S rRNA sequence within helix 44 of *M. smegmatis* wild-type and hybrid ribosomes after transplanting the A-site rRNA of eukaryotic ribosomes. (**A**) *Mycobacterium smegmatis*. (**B**) Human–bacterial hybrid ribosomes. (**C**) Hybrid ribosomal RNA containing the decoding-site rRNA of the protozoan *Leishmania*, which is also identical to *Trypanosoma*; and (**D**) *Blastocrithidia*. Base substitutions rendering the bacterial 16S rRNA eukaryotic are depicted in blue; the transplanted region is boxed. rRNA residues are numbered according to the nucleotide numbering used in *E. coli* 16S rRNA.

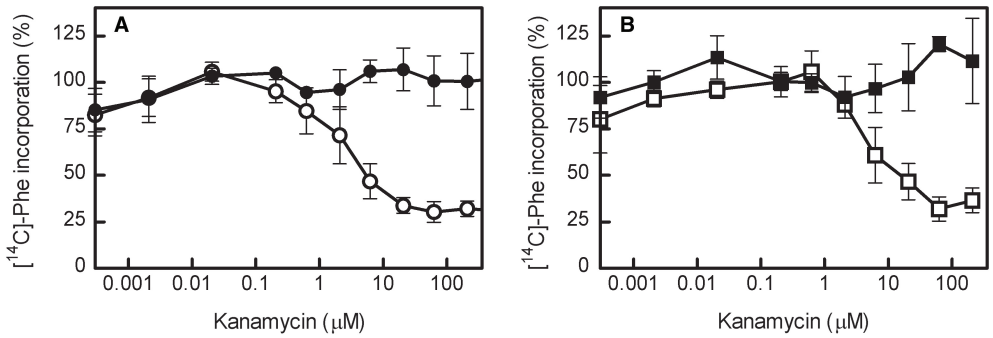

Figure 3. Kanamycin-induced inhibition of polypeptide synthesis using $(UUU)_{12}$-directed phenylalanine incorporation. (**A**) *Homo sapiens* cytosolic hybrid ribosomes (closed circles) versus *M. smegmatis* wild-type ribosomes (open circles). (**B**) *Leishmania* (closed squares) versus *Blastocrithidia* (open squares) hybrid ribosomes. The relative amount of [^{14}C]-phenylalanine incorporated by 5 pmol of purified 70S ribosomes after 60 min. incubation in the presence of varying concentrations of kanamycin A is shown. SEs are indicated. The corresponding IC_{50} values for kanamycin A and selected aminoglycoside antibiotics are given in Table 2.

4,5-disubstituted deoxystreptamines against *Blastocrithidia* hybrids were similar to those observed for the wild-type *M. smegmatis* strain (Table 1).

To analyze the antibiotic effects on translation in more detail, we determined the IC_{50} values for selected aminoglycosides in $(UUU)_{12}$-mRNA-directed phenylalanine incorporation in cell-free translation assays. *In vitro* translation assays were established with purified 70S ribosomes of wild-type and mutant *M. smegmatis* strains. A polyphenylalanine mRNA with a ribosomal-binding site, twelve consecutive (UUU) triplets coding for phenylalanine, and a stop codon was used to assess aminoglycoside-induced inhibition of [^{14}C]-phenylalanine incorporation in translating wild-type and hybrid ribosomes. Bacterial wild-type ribosomes were highly susceptible to all aminoglycosides tested, with IC_{50} values for poly-Phe synthesis below 3 μM (Figure 3 and Table 2). Hybrid ribosomes with a *Blastocrithidia* decoding region were likewise susceptible to low aminoglycoside concentrations. In contrast, hybrid ribosomes with the human decoding site were resistant, exhibiting IC_{50} values that are more than 500-fold higher than those observed in bacterial wild-type ribosomes. *Leishmania* hybrid ribosomes were resistant to all aminoglycosides tested except paromomycin. In order to assess aminoglycoside-stimulated misreading (25) in hybrid ribosomes, paromomycin was used

to measure drug-induced incorporation of leucine in a cell-free $(UUU)_{12}$ translation assay. In line with the other assays used to determine ribosomal drug susceptibility, the *Leishmania* hybrids were significantly more susceptible to paromomycin-induced mistranslation than the human hybrids (data not shown).

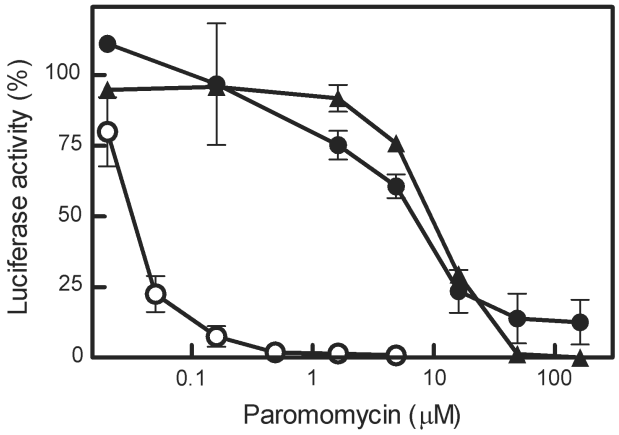

Figure 4. Paromomycin-induced inhibition of protein synthesis measured as luciferase activity in cell-free translation assays of firefly luciferase mRNA. Rabbit reticulocyte (closed triangles) versus human-bacterial hybrid (closed circles) and wild-type *M. smegmatis* (open circles) ribosomes; error bars represent the SEM ($n = 3$). The corresponding IC_{50} values for paromomycin and the aminoglycosides tested are given in Table 3.

Table 1. Activity of aminoglycoside antibiotics against cells carrying hybrid ribosomes

Compound	MIC (μg/ml)			
	Mycobacterium smegmatis	*Homo sapiens*	*Leishmania Trypanosoma*	*Blastocrithidia*
Paromomycin	1	≥1024	128	4
Neomycin	0.5	>1024	>1024	1
Geneticin	8	128	4	4
Gentamicin	1	>1024	>1024	1
Netilmicin	2	>1024	>1024	8
Tobramycin	1	1024	1024	2
Kanamycin A	1	>1024	>1024	1

We next wished to validate the hybrid ribosome approach by comparing the human–bacterial hybrid ribosomes to rabbit reticulocyte ribosomes (the decoding-site rRNA of human cytosolic ribosomes is identical to that of most vertebrates, including rabbit). In addition, with a view to define the selectivity of aminoglycoside compounds for protozoan versus human ribosomes more accurately, we wished to use an assay which is not limited by its sensitivity. Note that both the whole-cell determination of minimal inhibitory concentrations (MIC; Table 1) and the $(UUU)_{12}$ assay using purified ribosomes (Table 2) are limited by the amount of drug which can be applied. Towards this end we analyzed synthesis of luciferase based upon a coupled transcription–translation assay. Figure 4 shows the effect of paromomycin on protein synthesis in bacterial versus human–bacterial hybrid ribosomes and compared to rabbit reticulocyte ribosomes translating a luciferase mRNA. The paromomycin concentration required to inhibit synthesis of active luciferase in human-hybrid ribosomes to 50% (IC_{50}) closely resembled the IC_{50}

Table 2. Aminoglycoside-induced inhibition of $(UUU)_{12}$-directed phenylalanine incorporation

Compound	IC_{50} (μM)[a]			
	Mycobacterium smegmatis	*Homo sapiens*	*Leishmania Trypanosoma*	*Blastocrithidia*
Paromomycin	0.9	>500	96	2.7
Neomycin	0.4	>500	>500	0.7
Gentamicin	0.7	>500	>500	0.7
Netilmicin	0.7	>500	>500	1.1
Tobramycin	1.0	>500	>500	2.3
Kanamycin A	2.5	>500	>500	7.5

[a]Aminoglycoside concentrations required to inhibit [^{14}C]-phenylalanine incorporation to 50 percent (IC_{50}). Inhibition kinetics are exemplified by the graphs for kanamycin A presented in Figure 3. Best-fit nonlinear regression was used to define the 100% value and to calculate the IC_{50}. An antibiotic concentration of 1 μM corresponds to approximately four aminoglycoside molecules per ribosome.

Table 3. Aminoglycoside-induced inhibition of luciferase synthesis

Compound	IC_{50} (μM)[a]				
	Mycobacterium smegmatis	*Homo sapiens*	*Leishmania Trypanosoma*	*Blastocrithidia*	Rabbit reticulocyte lysate[b]
Paromomycin	0.03	4.7	0.18	0.05	9.2
Neomycin	0.04	13	6.7	0.05	18
Geneticin	0.03	0.8	0.02	0.02	0.2
Netilmicin	0.05	64	127	0.11	58
Tobramycin	0.02	21	60	0.05	38
Kanamycin A	0.05	116	204	0.03	67

[a]Aminoglycoside concentrations required to inhibit synthesis of active luciferase to 50 percent (IC_{50}). Inhibition kinetics are exemplified by the graphs for paromomycin presented in Figure 4. Best-fit nonlinear regression was used to define the 100% value and to calculate the IC_{50}.
[b]The rRNA decoding region of rabbit reticulocyte ribosomes is identical to that of human cytosolic ribosomes shown in Figure 2B.

in rabbit reticulocyte lysate. In fact, the IC_{50} values of the human hybrid ribosomes are virtually identical to the IC_{50} values determined for rabbit cytosolic ribosomes for all aminoglycosides tested in this study (Table 3). In contrast, the IC_{50} values in bacterial ribosomes were two to three orders of magnitude lower. Thus, transplanting helix 44 of the eukaryotic decoding region into bacterial ribosomes testified to be a valid approach for studying the specificity of aminoglycoside antibiotics, compounds that selectively affect prokaryotic versus eukaryotic ribosomes.

Analysis of aminoglycoside-induced inhibition of luciferase synthesis in *Leishmania* hybrid ribosomes and comparison to human hybrid ribosomes also allowed quantifying the differences in drug susceptibility. *Leishmania* hybrid ribosomes are ~20- to 30-fold more susceptible to the 4,5-disubstituted aminoglycoside paromomycin than human hybrid ribosomes. In line with the results from MIC determinations (Table 1) and aminoglycoside-induced inhibition of $(UUU)_{12}$-driven phenylalanine incorporation (Table 2), there is a significant difference in drug susceptibility between *Leishmania* hybrid and human hybrid ribosomes for aminoglycosides with a 6′-hydroxyl group. This is in contrast to aminoglycosides with a 6′-amino group where no such difference is present.

The basic mechanisms of translation of mRNA to protein are conserved throughout all organisms (17,26). Experimental data suggest that the selectivity of aminoglycoside antibiotics for bacterial ribosomes is due to defined phylogenetic variations of ribosomal RNA in an otherwise highly conserved binding site (13). The results obtained with the hybrid ribosomes are in excellent agreement with the drug susceptibilities determined in rabbit reticulocyte lysate (Table 3) and with data reported in the literature on complete eukaryotic ribosomes (25,27), demonstrating that the model system adequately reflects the situation in eukaryotic cytosolic ribosomes. Drugs targeting protein synthesis are infrequently used for treatment of infections with lower eukaryotes such as protozoan parasites, helminths or fungi, although the potential for selective activity appears to exist (28–33). Using various assays of drug activity, we demonstrated that the 4,5-disubstituted aminoglycoside paromomycin exhibits a distinct activity towards hybrid ribosomes carrying the cytosolic decoding site of *Leishmania* and *Trypanosoma*. These results provide an explanation for the activity of paromomycin in treatment of leishmaniasis (34,35), by pointing to the cytosolic ribosome as a drug target. Little treatment options involving quite toxic drugs, such as the trivalent arsenic compound melarsoprol, exist for late stage *T. brucei rhodiense* sleeping sickness (36). The availability of compounds with antitrypanosomal activity would be most significant, if only as second-line option for patients who experience relapse.

Defined point mutations have been introduced previously into the bacterial rRNA A site to study individual drug—nucleotide interactions of aminoglycosides (13). These studies demonstrated the role of nucleotides 1408, 1409 and 1491 in drug binding. A base substitution of adenine at position 1408 with a guanine interrupts the pseudo-base-pair formation of the adenine with ring I of the disubstituted 2-deoxystreptamines and alterations of the 1409–1491 bp interfere with the stacking interaction between 1491 and ring I. Our study complements this reductionistic approach by transferring the complete eukaryotic binding site into bacterial ribosomes. Given the complex structure of the ribosome and its various components that contribute to translational fidelity, e.g. ribosomal proteins S4, S5 and S12 (3), it is remarkable that a hybrid bacterial ribosome carrying the eukaryotic H44 shows a drug-susceptibility pattern identical to that of complete rabbit reticulocyte ribosomes. From this we conclude that additional phylogenetically variable ribosomal structures do not affect aminoglycoside susceptibility of the complete eukaryotic ribosome. In line with previous findings (13,37–39), a more general picture emerges, where ribosomes with a 1408 guanine in small subunit rRNA are resistant to aminoglycosides carrying an amino group at position 6′ of ring I (neomycin, gentamicin, netilmicin, tobramycin, kanamycin), while A sites with a non-canonical base-pair interaction between residues 1409 and 1491 are predominantly resistant to geneticin and paromomycin, drugs which carry a hydroxyl group at the 6′ position of ring I. Together, these results suggest that paromomycin and geneticin have a specific activity against ribosomes that are characterized by a canonical 1409–1491 bp; that neomycin, gentamicin, netilmicin, tobramycin and kanamycin are active against ribosomes with a 1408 adenine; and that metazoan cytosolic ribosomes are generally resistant to 2-deoxystreptamines.

Successful crystallizations of the ribosome and ribosome–drug complexes have initiated a rebirth of interest in the ribosome as drug target (40–43). Apparently, helix 44 of the rRNA decoding A site behaves as an autonomous domain, which can be dissected from the remaining part of the ribosome and shuffled between ribosomes of different phylogenetic domains *in vivo* while retaining its functional characteristics. It is likely that this possibility will help to study mechanisms of the various eukaryotic rRNA decoding sites in the future and to test hypotheses built upon X-ray structures. The construction of hybrid ribosomes, as demonstrated in this study, should also help characterizing the specificity of ribosomal inhibitors already available and of future compounds to come.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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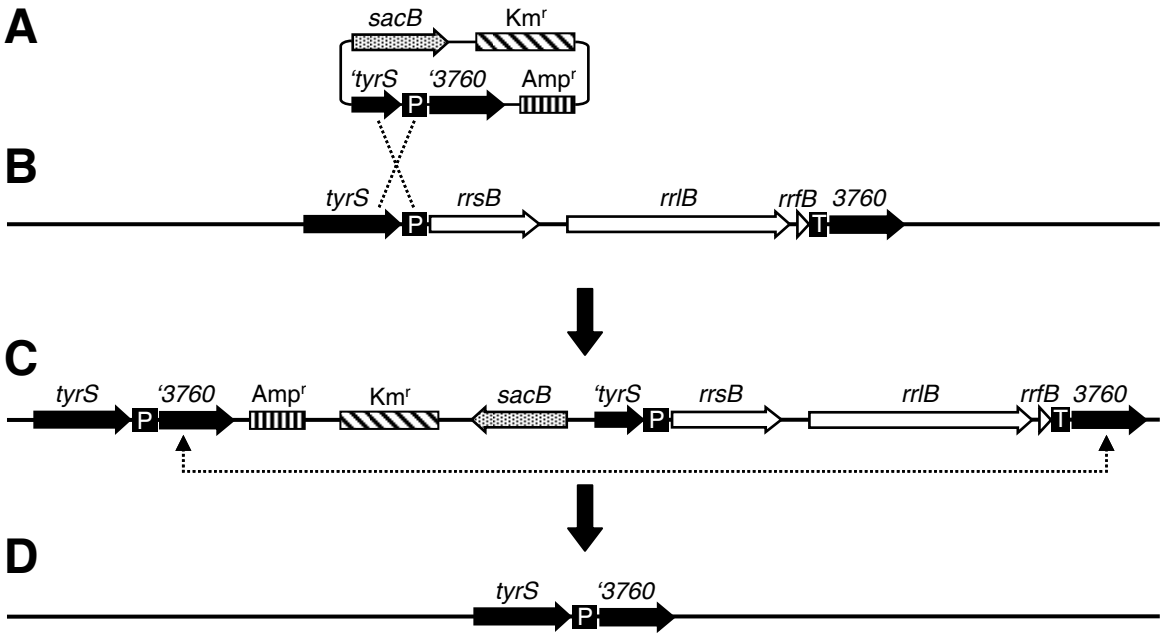
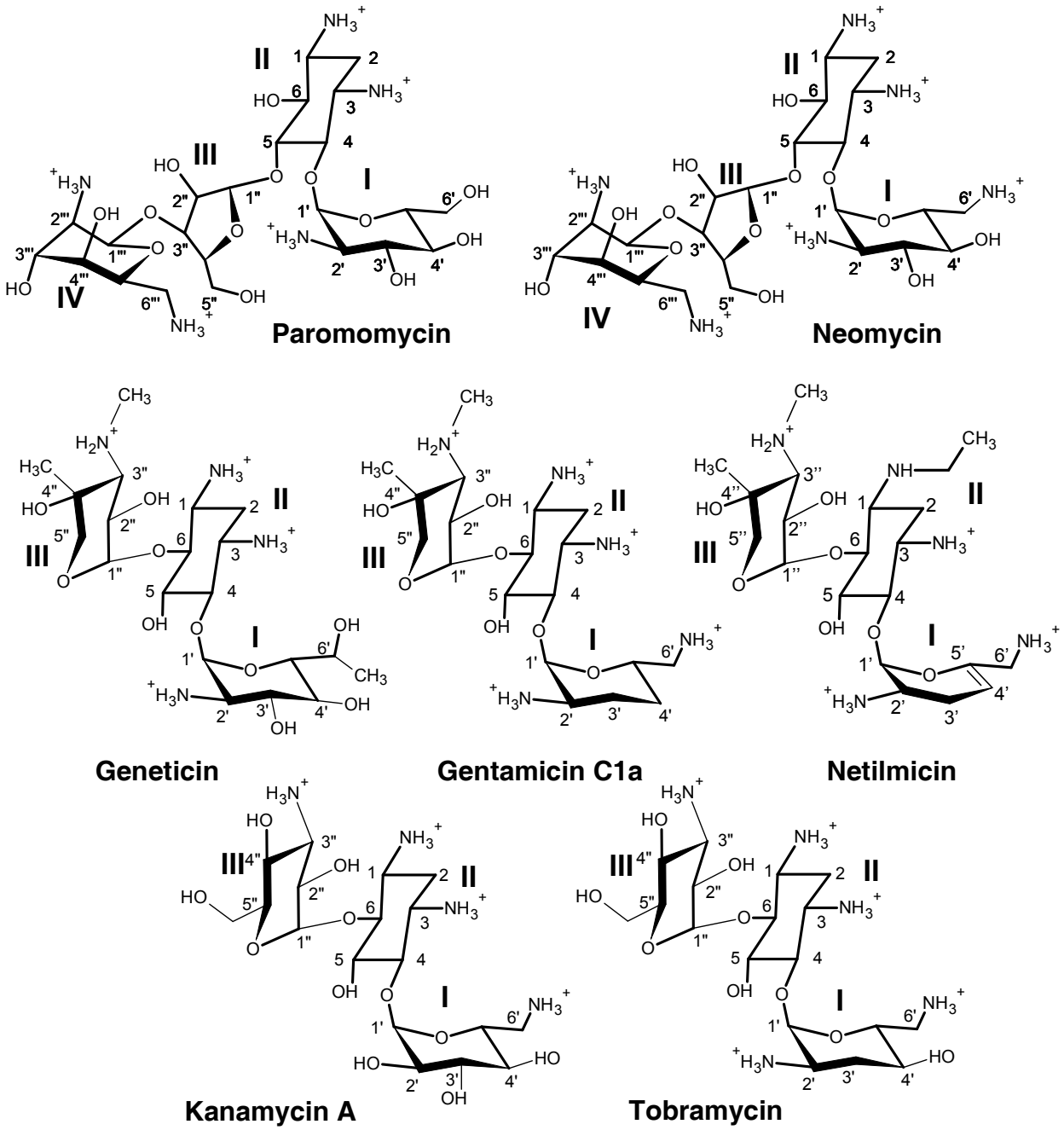


Supplementary Figure 1. Strategy for deletion of chromosomal rRNA operons. Broken lines indicate possible crossover sites between homologous sequences in the replacement vector (**A**) and the chromosomal target site (**B**). Following plasmid integration into the *rrnB* 5'-flanking region (**C**), a second crossover event between the homologous 3'-flanking sequences resolves the chromosomal tandem repeat to the deletion of *rrnB* (**D**). Open arrows represent rRNA genes; P and T the promoter and termination sequences, respectively. Solid arrows indicate the open reading frames upstream and downstream of *rrnB*. Hatched rectangles represent antibiotic resistance cassettes, the stippled arrow the *sacB* gene.



Supplementary Figure 2. Chemical structures of the disubstituted 2-deoxystreptamine antibiotics used in this study.

Genetic analysis of interactions with eukaryotic rRNA identify the mitoribosome as target in aminoglycoside ototoxicity

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Aminoglycoside ototoxicity has been related to a surprisingly large number of cellular structures and metabolic pathways. The finding that patients with mutations in mitochondrial rRNA are hypersusceptible to aminoglycoside-induced hearing loss has indicated a possible role for mitochondrial protein synthesis. To study the molecular interaction of aminoglycosides with eukaryotic ribosomes, we made use of the observation that the drug binding site is a distinct domain defined by the small subunit rRNA, and investigated drug susceptibility of bacterial hybrid ribosomes carrying various alleles of the eukaryotic decoding site. Compared to hybrid ribosomes with the A site of human cytosolic ribosomes, susceptibility of mitochondrial hybrid ribosomes to various aminoglycosides correlated with the relative cochleotoxicity of these drugs. Sequence alterations that correspond to the mitochondrial deafness mutations A1555G and C1494T increased drug-binding and rendered the ribosomal decoding site hypersusceptible to aminoglycoside-induced mistranslation and inhibition of protein synthesis. Our results provide experimental support for aminoglycoside-induced dysfunction of the mitochondrial ribosome. We propose a pathogenic mechanism in which interference of aminoglycosides with mitochondrial protein synthesis exacerbates the drugs' cochlear toxicity, playing a key role in sporadic dose-dependent and genetically inherited, aminoglycoside-induced deafness.

decoding | mitochondria | ribosomes | toxicity | translation

Low cost and high efficacy make aminoglycosides a common choice for treatment of serious infections caused by gram-negative bacilli, including endocarditis, sepsis, pneumonia, pyelonephritis, and multidrug-resistant tuberculosis (1). Unfortunately, aminoglycosides are both nephrotoxic and ototoxic. Although renal impairment is in general mild and reversible, ototoxicity results from drug-induced apoptosis of cochlear and vestibular hair cells and is irreversible (2, 3). Ototoxicity of aminoglycoside antibiotics occurs both in a dose-dependent and in an inherited idiosyncratic fashion. Despite attempts to limit drug doses and to monitor blood levels carefully, measurable signs of hearing loss are found in 20% of patients receiving aminoglycosides (2). Familial cases of aminoglycoside-induced deafness are maternally transmitted and linked to mutations in mitochondrial DNA (mtDNA) (4–6).

The mechanisms by which aminoglycoside antibiotics exert their toxic effects are controversial. A surprisingly large and diverse number of effects have been associated with aminoglycosides. Aminoglycosides have been reported to affect DNA, RNA, and protein synthesis; energy metabolism and ion transport; and synthesis or degradation of prostaglandins, gangliosides, mucopolysaccharides and lipids (2). In addition, it has been hypothesized that aminoglycosides may form cochleotoxic metabolites. Antioxidants apparently attenuate aminoglycoside-induced hearing loss, pointing to a role of the mitochondrion, an organelle involved in oxidation, as a target of ototoxic drugs (7, 8). Genetic analyses of individuals hypersensitive to aminoglycosides have identified mutations in mitochondrial rRNA. Transition mutations in the mitochondrial small ribosomal RNA gene, namely A1555G and, less frequently C1494T, have been identified as primary genetic traits in aminoglycoside-induced deafness (4, 6, 9). A1555G and C1494T both map to the aminoacyl-tRNA acceptor site (A site) of the small ribosomal subunit. The bacterial A-site rRNA is target for aminoglycoside antibiotics, which exert their antibacterial effect at the level of the prokaryotic ribosome (10–13). Aminoglycosides affect protein synthesis by inducing codon misreading and by inhibiting translocation of the tRNA-mRNA complex (14, 15). The basis for aminoglycoside selectivity is presumably their preferential binding to the bacterial as opposed to eukaryotic ribosomes (13, 16, 17).

The high copy number of mtDNA in mitochondria and the vast number of mitochondria in a single cell have frustrated any attempt of genetic manipulation of mitochondrial rRNA in lower and higher eukaryotes. Model oligonucleotides designed to mimic the drug-binding site have been used to investigate various aspects of aminoglycoside-ribosome interaction (18–22). However, conclusions derived from the study of model A-site oligonucleotides are compromised by several findings: (*i*) in contrast to drug susceptibility of complete ribosomes, binding affinities of aminoglycosides to prokaryotic decoding region constructs are not very sensitive to mutations within the RNA-binding region (23); (*ii*) *in vivo* drug susceptibilities of mutant ribosomes and *in vitro* binding affinities using variants of model A-site oligonucleotides may or may not correlate (24–26); (*iii*) the exquisite specificity of aminoglycosides for the prokaryotic as opposed to the eukaryotic cytosolic ribosome contrasts with the observation that these drugs bind to eukaryotic decoding-site constructs with approximately the same affinity as found for their prokaryotic counterpart (23, 24); and (*iv*) while there is evidence that mitochondrial ribosomes are susceptible to aminoglycosides (13, 27), oligonucleotides mimicking the mitochondrial A site do not bind aminoglycosides to any significant extent (24, 28).

Using gene-shuffling experiments, we have previously replaced the A-site residues of helix 44 (H44) in bacterial 16S rRNA with various eukaryotic homologues, demonstrating that the A-site rRNA behaves as an autonomous domain, which can be exchanged between different species for study of function (29,

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The authors declare no conflict of interest.

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Table 1. Minimal inhibitory concentrations (μg/ml) of aminoglycoside antibiotics

	A-site rRNA			
Aminoglycoside	Bacterial[a]	Mitochondrial (mt) hybrid	mt A1555G hybrid	mt C1494T hybrid
Neomycin B	0.5	16–32	8	8
Paromomycin	1	> 1,024	256–512	256–512
Kanamycin A	1	256–512	16–32	16
Tobramycin	1	128	16	16
Amikacin	0.5	32–64	2–4	2–4
Gentamicin	1	64–128	16–32	16–32
Netilmicin	2	512–1024	64	64–128

[a]*M. smegmatis* wild-type rRNA

30). Replacement of a 34-nucleotide portion of bacterial 16S-rRNA helix 44 with its human homologues resulted in rRNA-decoding sites virtually identical to that in cytosolic and mitochondrial ribosomes. Here we used hybrid bacterial ribosomes carrying distinct alleles of the mitochondrial decoding site to study aminoglycoside susceptibility of wild-type and mutant mitochondrial rRNA.

Results and Discussion

The *in vivo* activity of various 2-deoxystreptamine antibiotics against isogenic *Mycobacterium smegmatis* strains carrying mitochondrial-bacterial hybrid ribosomes was tested in minimal inhibitory concentration (MIC) assays, which determine growth inhibition at the whole-cell level. Compared to bacterial ribosomes, which were found to be unanimously susceptible to all aminoglycosides tested, the hybrid ribosomes with a wild-type mitochondrial H44 revealed a heterogeneous drug susceptibility pattern, with MIC values ranging from 32 to 1,024 μg/ml (Table 1). The ratio of MIC mitochondrial hybrid to MIC wild-type *M. smegmatis* varied from 64-fold (gentamicin, amikacin) to 256-fold (netilmicin, kanamycin), providing a relative measure of the drug-target selectivity of different 2-deoxystreptamine antibiotics. We next investigated recombinants where the bacterial H44 has been replaced by mitochondrial deafness alleles corresponding to mtDNA mutations A1555G and C1494T. The resulting mutant mitochondrial hybrid ribosomes differ from the wild-type mitochondrial hybrid only in 16S rRNA residues 1490 and 1410; compare Fig. 1*C* to Fig. 1 *D* and *E* (bacterial 16S rRNA residues are numbered according to *Escherichia coli* nomenclature). The presence of the A1555G or the C1494T mutation increased drug susceptibility of cells carrying the mitochondrial hybrid ribosomes by 4- to 16-fold (see Table 1).

For a more detailed study of the mt A1555G and C1494T alleles, we studied purified hybrid ribosomes in cell-free translation reactions. We first used an AUG(UUU)$_{12}$-mRNA template, as this message allows determination of drug-induced inhibition of polypeptide synthesis and amino acid misincorporation. Dose-response curves of aminoglycoside-induced inhibition of phenylalanine incorporation were analyzed to define the IC_{50} values of the individual 2-deoxystreptamines. Both the A1555G and the C1494T genotypes were more susceptible to aminoglycoside antibiotics than the wild-type mitochondrial decoding site, as indicated by the finding that significantly less drug concentrations were required to inhibit AUG(UUU)$_{12}$ mRNA-driven polyPhe synthesis [see Fig. 2*A*, Table 2, and supporting information (SI) Fig. S1].

Aminoglycosides are known to affect the translational fidelity of ribosomes by inducing misreading of the genetic code (14). For study of aminoglycoside-induced mistranslation we used the AUG(UUU)$_{12}$-driven polypeptide synthesis assay to determine

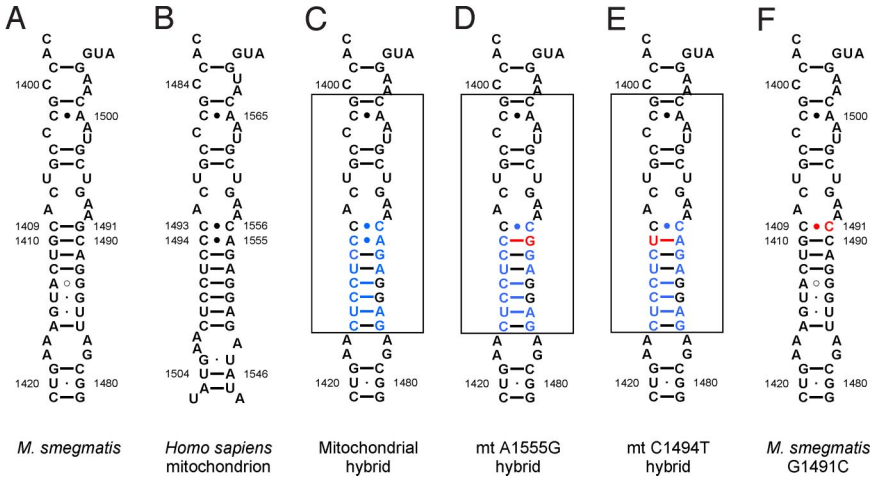

Fig. 1. Secondary structure of rRNA helix 44 in the ribosomal decoding site. (*A*) Decoding site of *M. smegmatis* wild-type ribosomes; rRNA nucleotides are numbered according to the bacterial nomenclature (i.e., homologous *E. coli* 16S rRNA positions). (*B*) Decoding site of human mitochondrial ribosomes; rRNA residues are numbered according to the mitochondrial nomenclature. (*C–E*) Mitochondrial decoding sites within human-bacterial hybrid ribosomes: wild type sequence (*C*) and deafness-associated alterations adenine to guanine at position 1490 (corresponding to mitochondrial mutation A1555G) (*D*); cytosine to uracil mutation at position 1410 (corresponding to mitochondrial mutation C1494T) (*E*). Nucleotide positions depicted in blue represent residues that are specific for human rRNA; nucleotide positions in red highlight the pathogenic mutations; the transplanted helix is boxed. (*F*) Decoding site of the *M. smegmatis* G1491C mutant.

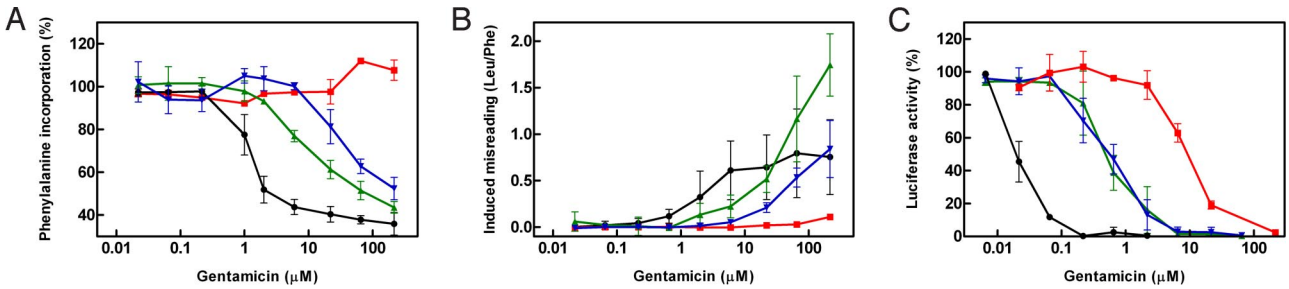

Fig. 2. Aminoglycoside susceptibility of mutant and wild-type mitochondrial hybrid ribosomes. Dose-response curves of wild-type mitochondrial (red squares), mutant A1555G (green triangles), and mutant C1494T (blue inverted triangles) hybrid ribosomes; bacterial ribosomes (black circles) are included for comparison. (*A*) Gentamicin-induced inhibition of [^{14}C]-phenylalanine incorporation ($n \geq 3$; ± SD.). The 100% value corresponds to 25 to 30 pmol Phe incorporation. Corresponding IC_{50} values of gentamicin and selected aminoglycoside antibiotics are presented in Table 2. (*B*) Gentamicin-induced increase in misincorporation of the near-cognate [^{3}H]-leucine relative to the drug-free control ($n \geq 3$; ± SD.). (*C*) Gentamicin-induced inhibition of luciferase synthesis relative to the drug-free control ($n \geq 3$; ± SD.). Corresponding IC_{50} values for gentamicin and selected aminoglycoside antibiotics are presented in Table 3.

the relative amount of near-cognate leucine incorporation compared to incorporation of the cognate amino acid phenylalanine in the presence of various concentrations of gentamicin. Relative incorporation of [^{3}H]-labeled leucine versus [^{14}C]-labeled phenylalanine was determined and plotted against gentamicin concentration. Introduction of the A1555G and C1494T alteration rendered the mitochondrial hybrid ribosomes highly susceptible to aminoglycoside-induced misreading (Fig. 2*B*; see also Fig. S2). In quantitative terms, the amount of gentamicin-induced misreading (calculated as leucine per phenylalanine incorporation) in A1555G and C1494T mutant hybrid ribosomes was up to 1.75 leucine per phenylalanine, as compared to a maximum of 0.2 leucine per phenylalanine for hybrid ribosomes with a wild-type mitochondrial decoding site.

To study the effect of aminoglycoside antibiotics on translation of a more natural mRNA template, we tested wild-type and mutant mitochondrial hybrid ribosomes in a cell-free luciferase synthesis assay. As depicted in Fig. 2*C*, Table 3, and Fig. S3, the allele- and drug-specific inhibition of luciferase synthesis essentially correlated with the results of the MIC and AUG(UUU)$_{12}$ assays. Drug-mediated inhibition of luciferase synthesis was significantly increased in A1555G and C1494T mutant ribosomes.

The basis for the selectivity of aminoglycosides is presumably their preferential binding to bacterial as opposed to eukaryotic ribosomes (10, 13, 16, 17). In particular, 16S rRNA nucleotides 1408, 1409, and 1491 of helix 44 have been shown to be critical for drug-binding by forming direct contacts with ring I of the 2-deoxystreptamines (12, 13, 16, 17, 26, 31–38) (see Fig. S4 for the chemical structures of aminoglycosides used in this study). In the absence of X-ray structures for aminoglycosides complexed to the mitochondrial ribosome, we can rationalize our findings on data invoked from the study of bacterial ribosome-drug complexes (12, 39). The rRNA secondary structure of the drug binding site in mitochondrial A1555G and C1494T mutant ribosomes resembles that of bacterial ribosomes with a G1491C alteration in that the C1409-C1491 opposition is accompanied by a 1410–1490 Watson–Crick pair (see Fig. 1). The bacterial G1491C ribosome shows a drug-susceptibility phenotype that is virtually superimposable on that found for the mitochondrial deafness alleles (see Table S1 for comparison). To determine whether the affinity of aminoglycosides to mutant mitochondrial decoding sites corresponds to that of the bacterial G1491C decoding site, we probed gentamicin binding by chemical footprinting experiments. Bacterial wild-type ribosomes showed drug-mediated protection from dimethyl sulfate (DMS) modification at G1405 (N-7), which is in good agreement with previous reports on aminoglycoside protection in bacterial 16S rRNA (34). Wild-type mitochondrial hybrid ribosomes showed little protection, while mutant mitochondrial A1555G and C1494T hybrid ribosomes showed a concentration-dependent protection of G1405 that resembles the dose-response curve observed with bacterial G1491C ribosomes (Fig. 3). Thus, binding of aminoglycosides to ribosomes with an adenine at 16S rRNA position 1408, appears to be mainly determined by the structural geometry of base pairs 1409–1491 and 1410–1490.

When studying drug-induced miscoding, we found that the decoding accuracy of bacterial G1491C ribosomes is barely affected by aminoglycoside antibiotics. In absolute terms and in contrast to the mitochondrial A1555G and C1494T deafness mutants, the bacterial G1491C ribosomes showed little drug-

Table 2. Aminoglycoside-induced inhibition of AUG(UUU)$_{12}$-driven phenylalanine incorporation (IC_{50}, μM)

	A-site rRNA			
Aminoglycoside	Bacterial[a]	Mitochondrial (mt) hybrid	mt A1555G hybrid	mt C1494T hybrid
Neomycin B	0.2 ± 0.0	209 ± 61	3.1 ± 0.3	3.7 ± 0.7
Paromomycin	0.7 ± 0.1	> 500	124 ± 35	116 ± 20
Kanamycin A	1.2 ± 0.2	> 500	20 ± 2	95 ± 29
Tobramycin	0.7 ± 0.1	> 500	15 ± 3	70 ± 23
Amikacin	0.8 ± 0.1	> 500	7.1 ± 0.8	13 ± 3
Gentamicin	1.3 ± 0.2	> 500	13 ± 2	62 ± 16
Netilmicin	1.2 ± 0.3	> 500	71 ± 10	331 ± 84

IC_{50} values represent the drug concentrations in μM that are required to inhibit AUG(UUU)$_{12}$-driven phenylalanine incorporation to half-maximal extent. A representative graph showing phenylalanine incorporation plotted against gentamicin concentration is shown in Fig. 2*A*.

[a]*M. smegmatis* wild-type rRNA

Table 3. Aminoglycoside-induced inhibition of luciferase synthesis (IC_{50}, μM)

	A-site rRNA			
Aminoglycoside	Bacterial[a]	Mitochondrial (mt) hybrid	mt A1555G hybrid	mt C1494T hybrid
Neamine	1.4	131	32	35
Neomycin B	0.04	0.7	0.5	0.4
Paromomycin	0.03	33	2.4	3.1
Kanamycin A	0.05	15.7	1.2	1.1
Tobramycin	0.02	7.8	0.8	0.9
Amikacin	0.02	7.0	0.4	0.6
Gentamicin	0.03	5.7	0.6	0.7
Netilmicin	0.05	17.6	0.8	2.6

IC_{50} values represent the drug concentrations in μM that are required to inhibit synthesis of functional firefly luciferase to 50%. Relative luciferase activity plotted against aminoglycoside concentration is shown in Fig. 2*C*.
[a]*M. smegmatis* wild-type rRNA

induced misreading (see Fig. S2). The bacterial G1491C mutant and the hybrid deafness ribosomes differ primarily in 16S rRNA residues 1413 to 1415 and 1485 to 1487, which form the lower stem of helix 44 (see Fig. 1). At the structural level, helix 44 interacts with helix 27. By modeling, nucleotide alterations in the lower stem of H44 have been suggested to affect this interaction and the relative movement between these two helices as part of the conformational change required in decoding (30). Apparently, the nature of the lower stem plays an important role in both spontaneous and drug-aggravated miscoding and determines the translational accuracy of the mutant decoding sites. Thus, susceptibility of A1555G and C1494T deafness mitoribosomes is the result of two mechanisms, which act in concert: increased drug binding to its target and excessive aggravation of the mutants' inherent deficiency in ribosomal accuracy.

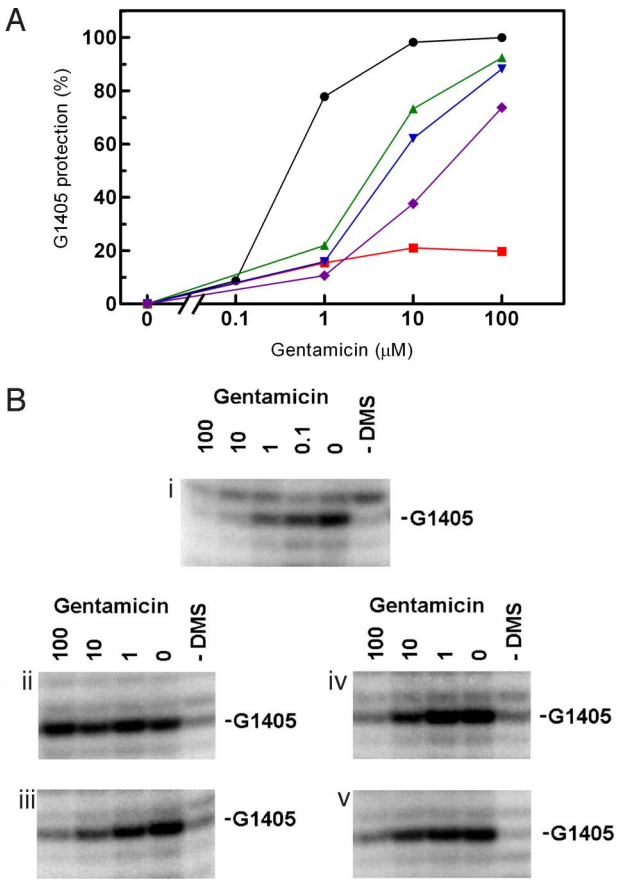

Fig. 3. Chemical footprints of gentamicin binding to wild-type and mutant mitochondrial decoding sites in comparison to bacterial wild-type and G1491C ribosomes. (*A*) Gentamicin-dependent protection of G1405 in wild-type mitochondrial (red squares), mutant mt A1555G (green triangles), mutant mt C1494T (blue inverted triangles), and bacterial G1491C (purple diamonds) decoding sites; wild-type bacterial ribosomes (black circles) are included for comparison. (*B*) Corresponding footprinting blots showing primer extensions starting with U1420. *i*, bacterial wild type; *ii*, mt wild type; *iii*, mt A1555G; *iv*, mt C1494T; *v*, bacterial G1491C.

Several lines of evidence link aminoglycoside ototoxicity to the mitochondrial ribosome: (*i*) mitochondrial ribosomes are structurally more similar to their prokaryotic ancestor than to the eukaryotic cytosolic homologues; (*ii*) compared to cytosolic ribosomes, the mitochondrial ribosomes of higher eukaryotes exhibit a remarkable degree of aminoglycoside susceptibility (27) [see Table S2, which compares the drug susceptibility of hybrid bacterial ribosomes with the A site (H44) of human cytosolic ribosomes to that of hybrid ribosomes with the mitochondrial decoding site]; and (*iii*) idiosyncratic drug susceptibility is associated with genetic predisposition, in particular mutations in mtDNA: 20 to 40% of patients with aminoglycoside-induced ototoxicity either carry the A1555G or the C1494T mutation in the 12S rRNA gene (6, 40).

To further assess whether aminoglycoside-induced ototoxicity is a result of the drugs' anti-mitoribosomal activity, we compared the potencies of a series of aminoglycosides to inhibit mitoribosome function with their relative cochleotoxicity in humans (41). The correlation between these two measures (Fig. 4) is consistent with the hypothesis that aminoglycoside-induced cochleotoxicity relates to the drugs' activity against mitochondrial ribosomes. Further evidence for this hypothesis is provided by our finding that netilmicin, which displays the least cochlear toxicity of the clinical aminoglycosides (reviewed in ref. 42), is significantly less active against hybrid mitochondrial ribosomes than gentamicin, tobramycin, or amikacin.

In summary, we provide experimental evidence for a mechanistic linkage between the mitochondrial A1555G and C1494T mutations and hypersusceptibility to aminoglycosides, although the exquisite tissue-specific action of aminoglycoside toxicity (that is, ototoxicity) is likely to involve additional factors (e.g., reactive oxygen species, drug uptake, or polyamine-like activation of NMDA receptors) (2, 43). Our results provide experimental support for aminoglycoside-induced dysfunction of the mitochondrial ribosome. We propose a pathogenic mechanism, in which interference of aminoglycosides with mitochondrial protein synthesis exacerbates the drugs' cochlear toxicity, playing a key role in sporadic dose-dependent and genetically inherited, aminoglycoside-induced deafness. Based upon our experiments, we suggest a scenario of aminoglycoside hearing

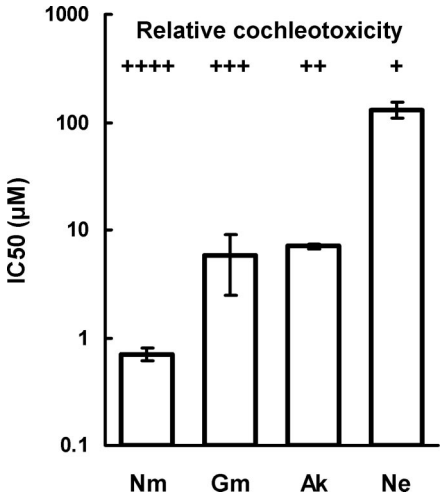

Fig. 4. Relationship between inhibition of protein synthesis in mitochondrial hybrid ribosomes and relative *in vitro* cochleotoxicity of aminoglycoside antibiotics. The potencies of a series of cochleotoxic aminoglycosides (Ak, amikacin; Gm, gentamicin; Ne, neamine; Nm, neomycin) in inhibiting protein synthesis in hybrid mitochondrial ribosomes (see Table 3) correlates with the relative cochleotoxicity previously reported by Kotecha and Richardson (41).

loss, which is initiated by mitoribosomal misreading, subsequently via activation of downstream signaling pathways, such as MAPK and JNK (44, 45), misreading results in hair cell death through apoptosis.

Materials and Methods

Construction of Mutant Strains with Hybrid Ribosomes. The recently described *M. smegmatis* mc^2 155 Sm^5 Δ*rrnB* (38) was used for all genetic manipulations. Site-directed mutagenesis of its single rRNA operon was done by PCR mutagenesis using hybrid rDNA oligonucleotides comprising the wild-type or mutant mitochondrial helix 44 decoding-site sequence. The resulting hybrid gene fragment was cloned into an integration-proficient plasmid used to transform *M. smegmatis* Δ*rrnB*. Transformants were selected on LB agar plates containing 20 μg/ml paromomycin for gene replacement by homologous recombination. Resulting recombinant *M. smegmatis* cells had the central 34-nucleotide part of the bacterial H44 replaced by its mitochondrial counterpart. Successful replacement of the bacterial decoding-site sequence with the mitochondrial sequence was controlled by sequence analysis of the chromosomal *rrnA* locus.

Minimal Inhibitory Concentration Assays. Minimal inhibitory concentrations of neomycin B, paromomycin, kanamycin A, tobramycin, amikacin, gentamicin, netilmicin (all Sigma), and neamine were determined by broth microdilution assays as described previously (46). Neamine was a kind gift of Andrea Vasella, ETH Zurich. The gentamicin used in this study is a mixture of gentamicin C_1, gentamicin C_{1a}, and gentamicin C_2 in a 45:35:30 ratio.

Isolation and Purification of Ribosomes. Ribosomes were purified from bacterial cell pellets as described previously (30). In brief, ribosome particles were isolated by successive centrifugations and fractionated by sucrose gradient (10–40%) centrifugation. The 70S ribosome-enriched fraction was pelleted, resuspended in association buffer, incubated for 30 min at 4 °C, dispensed into aliquots, and stored at −80 °C following shock freezing in liquid nitrogen. Ribosome concentrations of 70S were determined by absorption measurements on the basis of 23 pmol ribosomes per A_{260} unit. Integrity and functional activity of purified 70S ribosomes was determined by analytical ultracentrifugation and by assessing their capacity to form initiation complexes, as described previously (29).

Cell-free AUG(UUU)$_{12}$ Translation Assays. Cell-free translation reactions were done as described previously (30). A reaction mixture containing *M. smegmatis* tRNA^{bulk}, amino acids, S100 extract, energy mix, pyruvate kinase, and polyamines was preincubated with 30 μM [^{14}C]-phenylalanine (110 mCi/mmol) and/or 30 μM [^{3}H]-leucine (500 mCi/mmol) at 37 °C for 15 min. The translation reaction was started by addition of ribosomes to a final concentration of 0.25 μM, AUG(UUU)$_{12}$-mRNA (5′-GCGGCAAGGAGGUAAAUA *AUG* (UUU)$_{12}$ *UAA* GCAGG-3′, obtained from Dharmacon) to 1 μM, and aminoglycoside antibiotics in serial dilutions. Following incubation for 60 min at 37 °C, the reaction was stopped by addition of KOH, precipitated polypeptides were collected on filters, and [^{14}C]-phenylalanine or [^{3}H]-leucine were quantified. Background values for Phe and Leu incorporation were 0.4 to 0.5 pmol at time zero; the background was not subtracted from the experimental values determined.

Cell-Free Luciferase Translation Assays. Purified 70S hybrid ribosomes were used in a coupled transcription-translation reaction as described previously (29). The reaction mixture was incubated for 60 min at 37 °C, stopped on ice, and luciferase assay substrate (Promega) was added. Functional protein was quantified by measuring bioluminescence in a luminometer (Bio-Tek instruments, FLx800).

Footprinting Analyses. DMS modification of 70S ribosomes (20 pmol) was performed in 100 μl buffer containing 80 mM potassium cacodylate (pH 6.5), 100 mM ammonium chloride, 20 mM magnesium chloride, 1 mM DTT, and 0.5 mM EDTA. Following ribosome activation for 15 min at 37 °C, gentamicin was added and the reaction mixture was incubated for another 15 min before addition of DMS (6 μl, 1:10 in ethanol). Following a 30-min incubation at 37 °C, the reaction was stopped by addition of 100 μl DMS Stop solution (50 mM Tris, pH 7.5, 300 mM sodium chloride, 1% SDS, 200 mM β-mercaptoethanol). Ribosomes were precipitated with ethanol, pelleted, resuspended in 200 μl 50 mM Tris pH 8, 0.5% SDS, and extracted with phenol/chloroform. DMS-modified RNA was precipitated with ethanol and sodium borohydride reduction and aniline-induced strand scission was performed as described previously (47). Primer extension of 16S rRNA was performed as described (48), using DNA oligonucleotides complementary to 16S-rRNA nucleotides 1445 to 1421. Air-dried gels were scanned and quantified using the STORM PhosphorImaging System with ImageQuant 5.2 Software (Amersham Bioscience).

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Supporting Information

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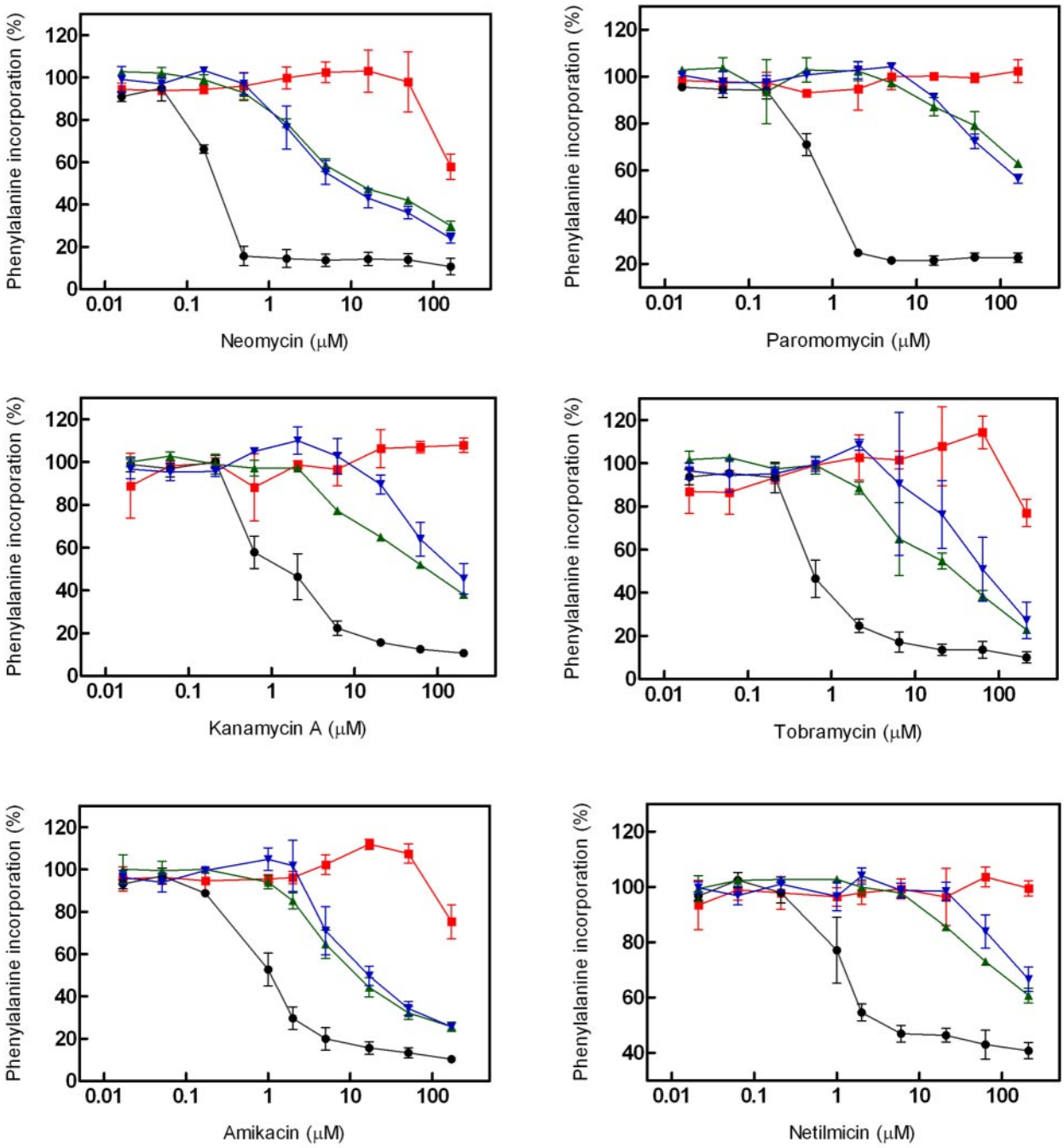

Fig. S1. Aminoglycoside susceptibility of bacterial hybrid ribosomes in AUG(UUU)$_{12}$-directed polypeptide synthesis. Dose-response curves of bacterial (black circles), mitochondrial (red squares), mutant A1555G (green triangles), and mutant C1494T (blue inverted triangles) hybrid ribosomes. Corresponding IC_{50} values are given in Table 2.

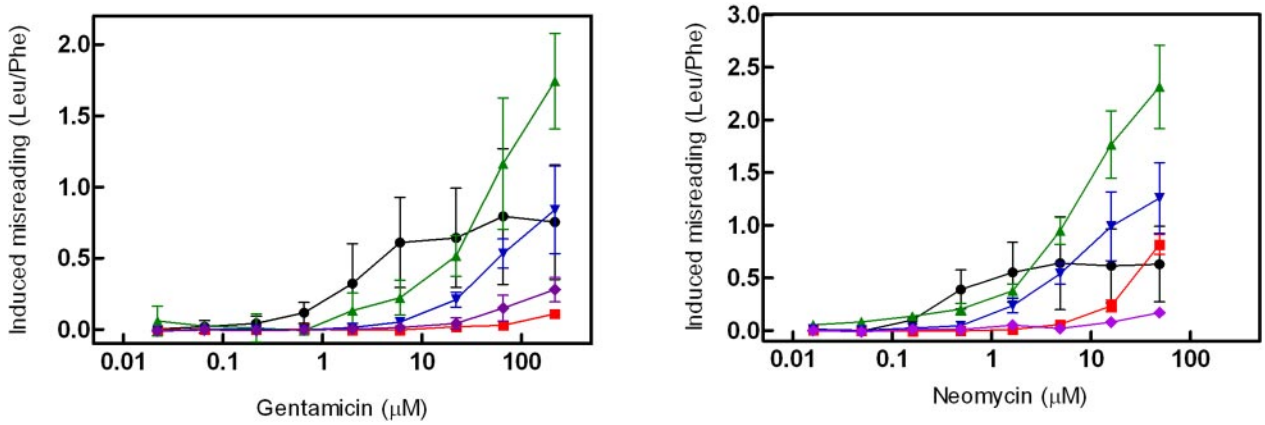

Fig. S2. Aminoglycoside-induced miscoding relative to a drug-free control in a *M. smegmatis* G1491C mutant in comparison to mutant mitochondrial decoding sites. Dose-response curves of *M. smegmatis* G1491C (purple diamonds), mitochondrial (red squares), mutant A1555G (green triangles), and mutant C1494T (blue inverted triangles) hybrid ribosomes. Bacterial wild-type ribosomes (black circles) are included for comparison.

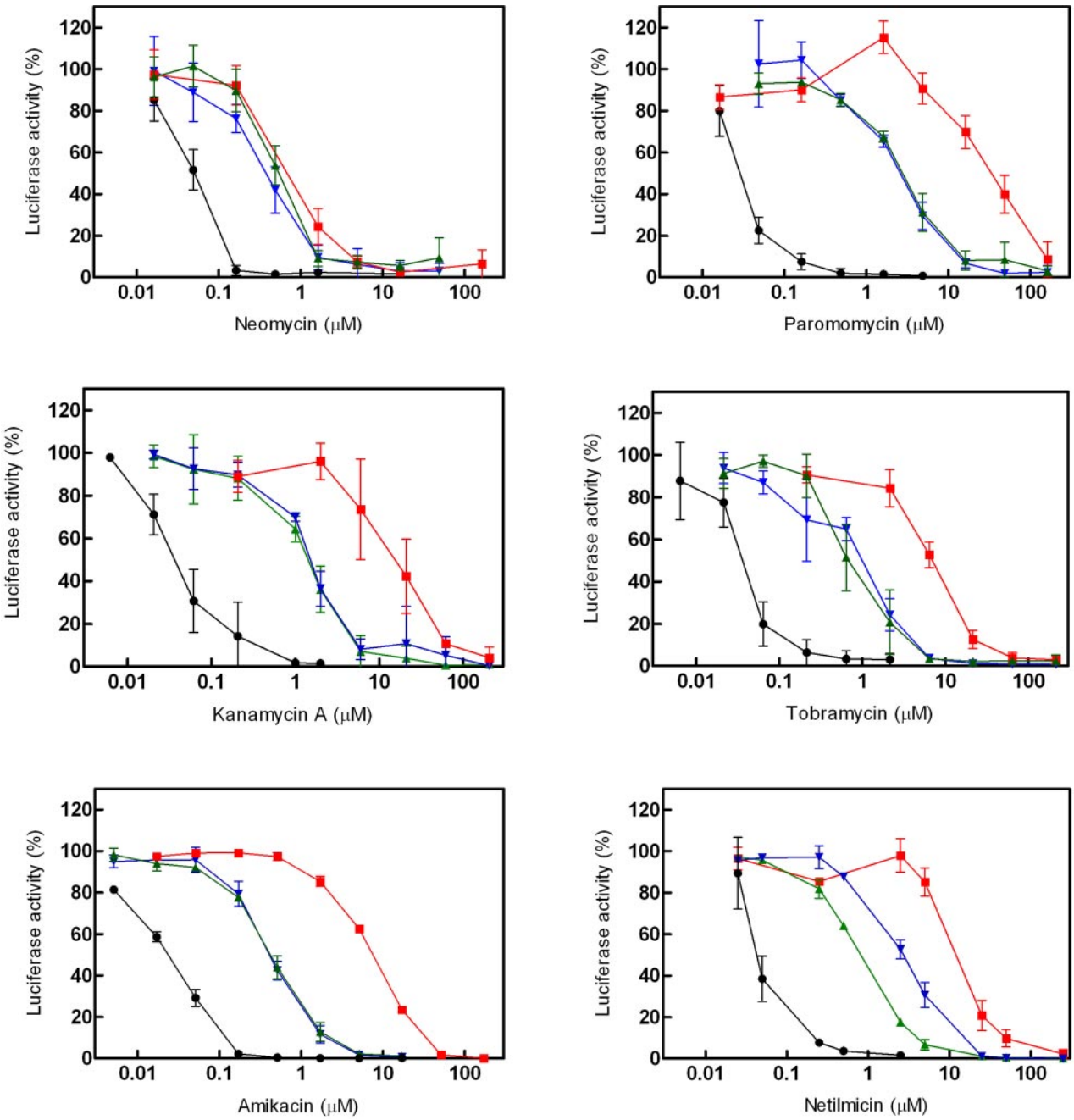

Fig. S3. Aminoglycoside-induced inhibition of luciferase synthesis. Dose-response curves of bacterial (black circles), mitochondrial (red squares), mutant A1555G (green triangles), and mutant C1494T (blue inverted triangles) hybrid ribosomes. Corresponding IC_{50} values are given in Table 3.

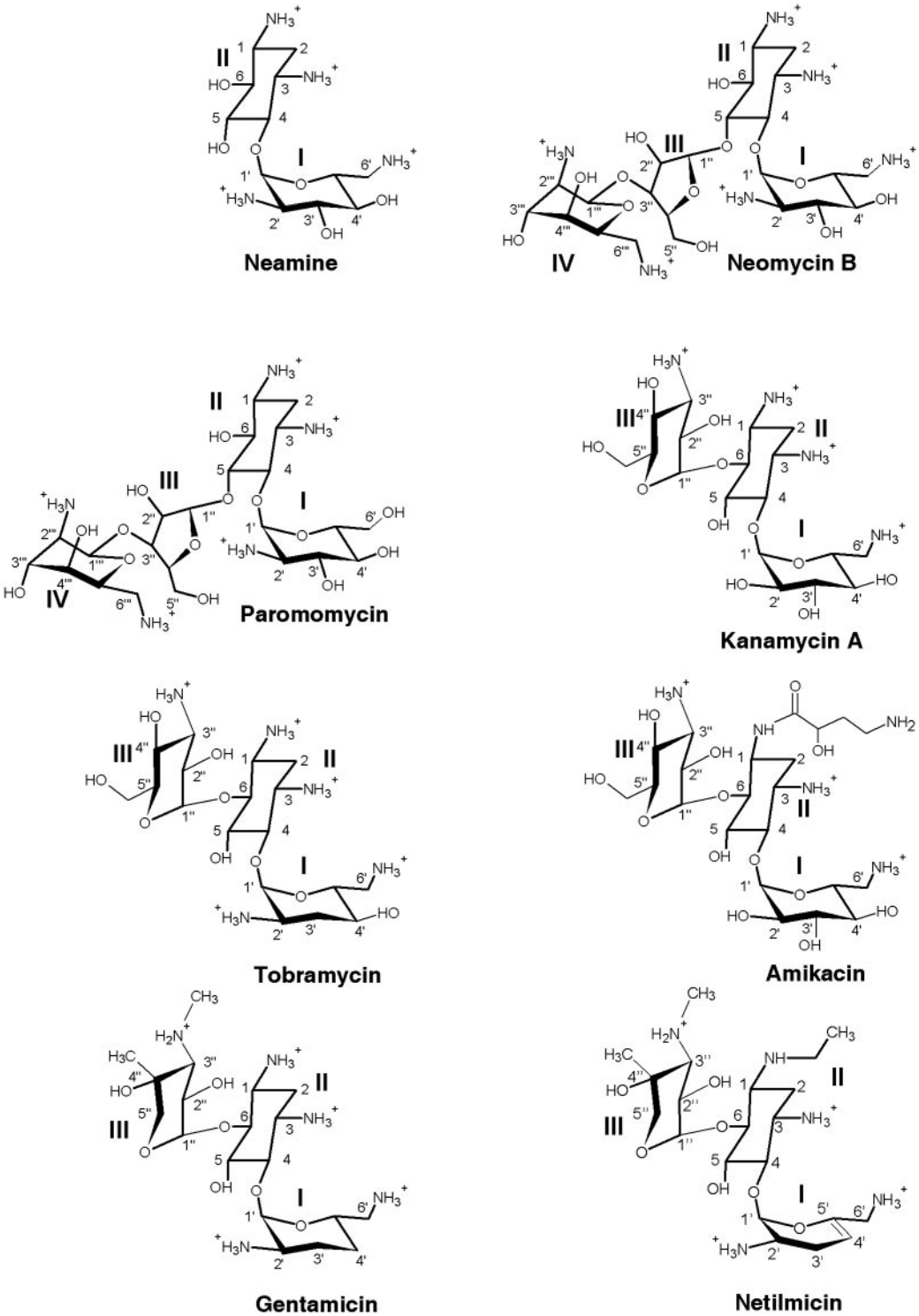

Fig. S4. Chemical structures of 2-deoxystreptamine antibiotics used in this study.

Table S1. Minimal inhibitory concentrations (μg/ml) against *M. smegmatis* G1491C mutant in comparison to mutant mitochondrial hybrids

	A-site rRNA				
	M. smegmatis	Mitochondrial (mt) hybrid	mt A1555G hybrid	mt C1494T hybrid	*M. smegmatis* G1491C
Neomycin B	0.5	16–32	8	8	16
Paromomycin	1	> 1,024	256–512	256–512	512
Kanamycin A	1	256–512	16–32	16	16–32
Tobramycin	1	128	16	16	16
Amikacin	0.5	32–64	2–4	2–4	4
Gentamicin	1	64–128	16–32	16–32	16–32
Netilmicin	2	512–1,024	64	64–128	128

Table S2. Aminoglycoside susceptibility of the mitochondrial versus the cytosolic decoding site

	MIC (μg/ml)	
	Mitochondrial hybrid	Cytosolic hybrid
Neomycin B	16–32	> 1,024
Kanamycin A	256–512	> 1,024
Tobramycin	128	1,024
Amikacin	32–64	512–1,024
Gentamicin	64–128	> 1,024
Netilmicin	512–1,024	> 1,024

(Manuscript in preparation)

Mutation K42R in Ribosomal Protein S12 Does Not Affect Susceptibility of *Mycobacterium smegmatis* 16S rRNA A- site Mutants to 2-deoxystreptamine Aminoglycosides.

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Abstract

Recent studies have suggested that ribosomal protein S12 modulates 16S rRNA function and susceptibility to 2-deoxystreptamine aminoglycosides. To study whether the non-restrictive K42R mutation in RpsL affects 2-deoxystreptamine susceptibility in *Mycobacterium smegmatis*, we studied the drug susceptibility pattern of various mutants with genetic alterations in the 16S rRNA decoding A-site in the context of wild-type and mutant protein S12. RpsL K42R substitution was found not to affect the drug resistance pattern associated with mutational alterations in 16S rRNA H44.

Introduction

Ribosomal protein S12 is a critical component of the A-site of the 30S ribosomal subunit and is involved in both tRNA selection and resistance to streptomycin [1,2]. Mutations in *rpsL* coding for ribosomal protein S12 are known to affect ribosomal accuracy to various extents, resulting in what is characterized as error-restrictive or non-restrictive S12 alterations [3]. Streptomycin inhibits protein synthesis and makes ribosomes error prone by affecting initial tRNA selection and proof-reading [4]. Mutations in S12 confer streptomycin resistance by preventing streptomycin binding and/or conferring ribosomal hyperaccuracy; a strongly hyperaccurate phenotype may even manifest as streptomycin-dependence [2]. Various substitutions at positions 42 and 87 are associated with streptomycin resistance. In particular, mutations Lys42 → Arg, Ala or Thr promote high levels of streptomycin resistance; among those Lys42Arg has a non-restrictive phenotype [3], whereas Lys42Ala and Lys42Thr are strongly error-restrictive [3,5,6]. S12 substitutions Lys87 → Gln or Gly confer different degrees of streptomycin resistance [7,8]. The homologous mammalian ribosomal proteins

carry Gln-87 (human mitochondrial ribosomes) or Gly-87 (human cytoplasmic ribosomes) in part accounting for the drugs' prokaryotic specificity [7].

More recently it has been suggested that ribosomal protein S12 modulates 16S-rRNA function and susceptibility to 2-deoxystreptamine aminoglycosides [5,6]. 2-deoxystreptamine aminoglycosides are composed of a common core, termed neamine, in which position 4 of a 2-deoxystreptamine ring (ring II) is attached to a glycopyranosyl ring (ring I). Additional sugars are attached to position 5 or 6 of the 2-deoxystreptamine moiety to give rise to 4,5- or 4,6- aminoglycosides (see Supplementary Data Fig. S1). In 4,5- aminoglycosides, the core is further substituted by one (ribostamycin), two (neomycin, paromomycin) or three (lividomycin) additional sugars attached to position 5 of ring II, whereas in 4,6-aminoglycosides (gentamicin, tobramycin, kanamycin, etc.) the core is further substituted by one additional sugar attached to position 6 of ring II. The drug binding pocket for these compounds consists of an internal loop of 16S-rRNA helix 44 – the decoding A-site of the ribosome [9]. We have previously performed extensive genetic studies of 16S-rRNA helix 44 in *Mycobacterium smegmatis* to address the role of individual rRNA residues in drug binding (reviewed in [10]). These studies have been conducted mainly in the genetic background of a non-restrictive K42R mutation in ribosomal protein S12 [10-16]. The K42R mutation confers high-level resistance to streptomycin and was used as counter-selectable marker in strain construction [15]. The recent reports on the interplay of S12 on 16S rRNA function and susceptibility to 2-deoxystreptamine aminoglycosides prompted us to study in detail the role, if any, between RpsL K42R and mutational alterations in 16S-rRNA helix 44 conferring resistance to 2-deoxystreptamines. We find that the non-restrictive RpsL K42R mutation does not affect the 2-deoxystreptamine susceptibility of various rRNA mutations in *M. smegmatis* H44.

Results and Discussion

Generation of *M. smegmatis* strains with mutations in S12 and 16S rRNA

M. smegmatis was the first eubacterial model organism that was made single rRNA allelic by means of deletion mutagenesis and that allowed for genetic studies of its ribosomal RNA [16]. As a gram-positive mesophilic bacterium, *M. smegmatis* is susceptible to a number of ribosomal antibiotics and sets itself apart from other model organisms by being a close representative of clinically relevant pathogenic bacteria. Early genetic studies on susceptibility to 2-deoxystreptamine aminoglycoside antibiotics relied on *M. smegmatis* strains with a K42R mutation in ribosomal protein S12 [10-16]. To substantiate the results and conclusions drawn from these early studies, we constructed a comprehensive set of *M. smegmatis* rRNA mutants in the context of both a wild-type and a mutant S12 (Table S1).

The set of 16S rRNA mutants includes alterations of positions 1408 (A1408G), 1491 (G1491C; G1491U; G1491A) and 1409 (C1409U; C1409G).

Aminoglycoside susceptibility of *M. smegmatis* mutants

Aminoglycoside susceptibility of the mutant strains was determined by minimal inhibitory concentration (MIC) assays. Representatives for each of the two disubstituted 2-deoxystreptamine subclasses were included: the 4,5-disubstituted 2-deoxystreptamines paromomycin and neomycin and the 4,6-disubstituted 2-deoxystreptamines gentamicin, tobramycin and kanamycin. The results are presented in Table 1.

Table 1. Drug susceptibility of *M. smegmatis* 16S rRNA mutants: wild-type S12 versus K42R

16S rRNA residues		S12	MIC (µg/mL)					Reference
1408	1409–1491		Pm	Nm	Gm	Tb	Km	
A	C≡G	wt	1	0.5	1	1	0.5-1	this study
A	C≡G	K42R	1	1	1	1	0.5-1	[12]
G	C≡G	wt	64	> 1024	> 1024	> 1024	> 1024	this study
G	C≡G	K42R	64	≥ 1024	> 1024	> 1024	> 1024	[12]
A	C • C	wt	512	16	16-32	16	16-32	this study
A	C • C	K42R	512	16-32	16-32	16-32	16-32	[12]
A	C • U	wt	512-1024	8-16	32-64	64	64-128	this study
A	C • U	K42R	512	8-16	32	32-64	128	[12]
A	C • A	wt	32-64	2	2	2	1-2	this study
A	C • A	K42R	32	4	2	4	2	[12]
A	U · G	wt	4-8	0.5-1	8	8-16	8-16	this study
A	U · G	K42R	4-8	1	8-16	8-16	16	[12]
A	G • G	wt	32-64	4-8	1-2	8-16	32-64	this study
A	G • G	K42R	16-32	4	2-4	8-16	16-32	[12]

Pm, paromomycin; Nm, neomycin; Gm, gentamicin; Tb, tobramycin; Km, kanamycin A.

Among all A-site mutations that confer aminoglycoside resistance, the A1408G mutation is the most significant. In *M. smegmatis* the A1408G mutation confers moderate resistance to paromomycin (6′ OH) but high level resistance to 2-deoxystreptamine aminoglycosides with an amino group at the 6′ position of ring I. A key element in drug binding is the pseudo base-pair interaction between the aminoglycosides' ring I and A 1408 [13,17,18]. In case of an adenine, the oxygen of ring I accepts a hydrogen bond from the N6 of A1408, and the amino or hydroxyl group at position 6′ donates a hydrogen bond to the N1 of adenine, accounting for two direct hydrogen bonds between ring I and A1408 (Fig. 1). In case of a 1408 guanine, the 6′ amino group of ring I can no longer form an H bond with the Watson-Crick edge of residue 1408. Additionally, the positive charge of the 6′ amino group creates repulsion against the N1 and N2 amino groups of guanine. As a consequence, the A to G mutation prevents aminoglycoside binding by precluding the proper insertion of ring I into the binding site. In contrast, a 6′- hydroxyl group, as in paromomycin could still become an acceptor of an H bond from N1 or N2 of the guanine, although the resulting pseudo base pair does not appear to promote optimal insertion of ring I, as indicated by decreased ribosomal drug susceptibility.

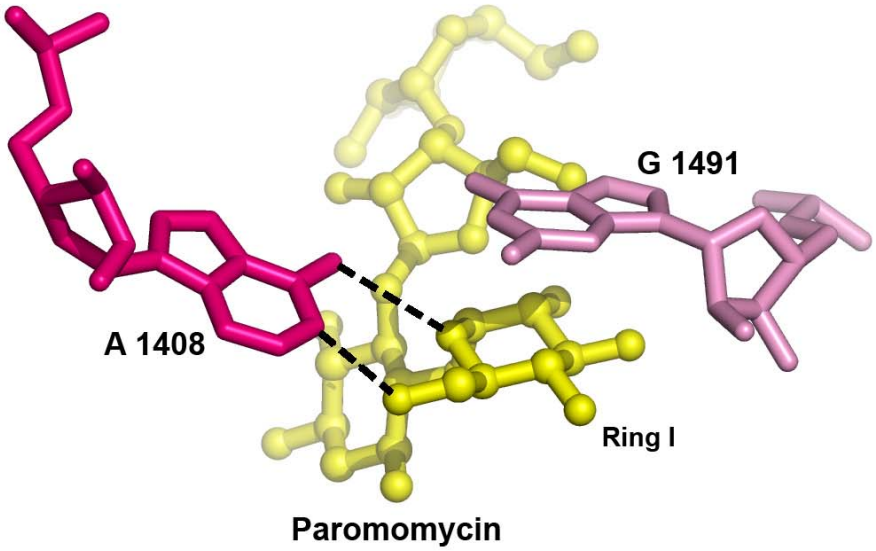

Figure 1. Stacking interaction of ring I with G1491 and pseudo-base pairing of ring I with A1408. The hydrogen bonding contacts between ring I and A1408 are indicated by black broken lines

The Watson–Crick base pair C1409–G1491 forms the base of the drug binding pocket. In the crystal structures [17,18], G1491 provides a stacking interaction with ring I of the aminoglycosides, thereby stabilizing the pseudo base-pair interaction of ring I with A1408 (Fig. 1). Among all mutations investigated affecting base-pair interaction C1409-G1491, the transversion mutations G1491C and G1491U (resulting in pyrimidine-pyrimidine oppositions) confer the highest level of resistance, in particular to paromomycin. Presumably, a pyrimidine-pyrimidine opposition provides a conformation that sterically hinders the correct positioning of ring I. A pyrimidine-purine opposition is retained following transition of G1491 to A resulting in 1409C–A1491. This mutational alteration apparently interferes less with drug binding, as indicated by the mutants' drug susceptibility pattern. The C1409U mutant shows little resistance to aminoglycosides. Nucleotide C1409 is not involved directly in drug binding, but is responsible for the correct orientation of nucleotide 1491, as 1409 and 1491 form a Watson–Crick base pair. The mutant wobble-base pair interaction 1409U–G1491 is likely to show conformational characteristics resembling those of the wild-type C–G. Mutation C1409G leads to a purine–purine opposition 1409G–G1491 in which the exact nature of interaction is difficult to predict. Surprisingly, the resistance levels conferred by this mutation are low to moderate. In general, sequence alterations in C1409-G1491 while mostly affecting the 6′ OH paromomycin do not discriminate between 4,5- and 4,6-aminoglycosides [10]. This is in agreement with the structural observation that ring I binds in the same orientation irrespective of the substituents at the 2-deoxystreptamine ring [18].

Strains with mutations in 16S rRNA residues 1408, 1409 and 1491 show a mutation-specific drug susceptibility pattern that was independent of the amino acid residue 42 in ribosomal protein S12 (wt *vs* K42R, see Table 1) and that corresponded to previously published data [12]. These results demonstrate that the K42R mutation in ribosomal protein S12 does not affect the susceptibility of *M. smegmatis* H44 mutants to 2-deoxystreptamines. Notably, this finding is not fully congruent with a previous finding in *E. coli* where K42R (the *rpsL*226 allele) reportedly modulated the level of paromomycin resistance of a G1491U mutant [19].

To study whether the different findings reported for *E. coli* are due to phylogenetic differences in the decoding-site rRNA, we constructed *M. smegmatis* with a proteobacteria-like helix 44 (Fig. 2). *M. smegmatis* and *E. coli* differ in 16S rRNA residues 1410-1490 and 1411-1489: 1410-1490 G-C (*M. smegmatis*) *versus* A-U (*E. coli*), 1411-1489 U-A (*M. smegmatis*) *versus* C-G (*E. coli*). We found that the various H44 mutations (A1408G, G1491A, G1491C, G1491U) resulted in identical drug susceptibility patterns regardless of a mycobacterial and proteobacterial H44 sequence context at residues 1410-1490 and 1411-1489 (data not shown).

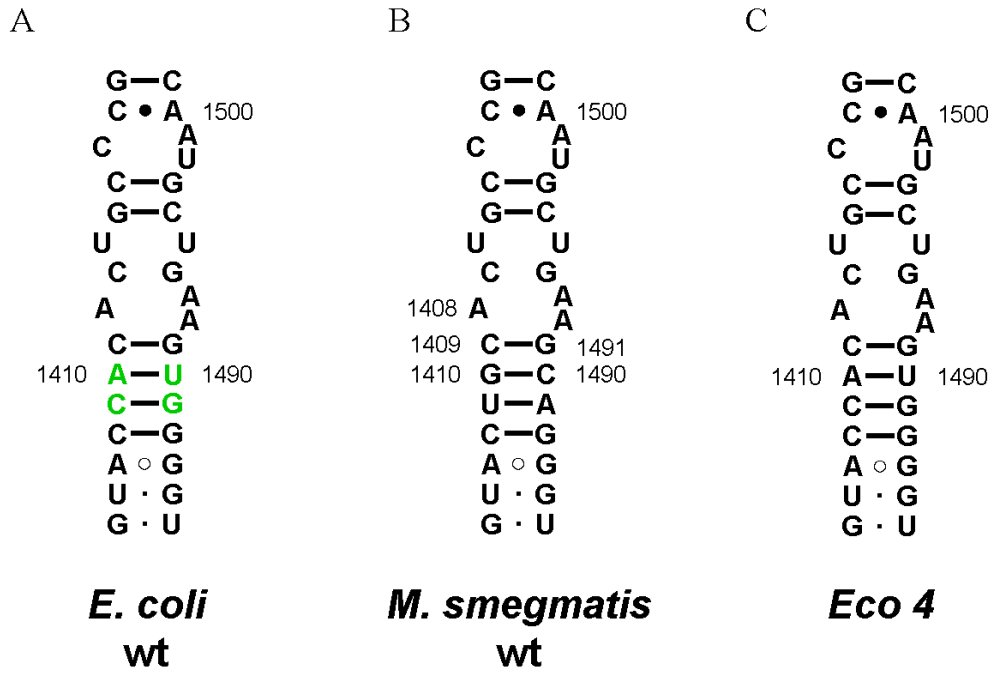

Figure 2. Secondary structure of 16S rRNA helix 44 in the ribosomal decoding site. (**A**) Decoding site of *E. coli* wild-type (Proteobacteria). Four nucleotide positions depicted in green represent residues that are specific for *E. coli* 16S rRNA. (**B**) Decoding site of *M. smegmatis* wild-type (Mycobacteria) (**C**) Decoding site of *M. smegmatis* mutagenised to correspond to the polymorphism observed in *E. coli* i.e., Eco4.

Structural analysis of K42 mutations

Crystal structures of streptomycin bound to the small ribosomal subunit of *T. thermophilus* [17] have revealed two direct hydrogen bonds between streptomycin and the lysine residue 42 of ribosomal protein S12 (Fig. 3A, 3B). K42 forms an additional contact to the phosphate backbone of 16S-rRNA helix 27 (H27) via a salt bridge to the phosphate group of residue A913. Superimposition of the K42R substitution disrupts the hydrogen bonding to streptomycin (Fig. 3C), accounting for the streptomycin resistance of K42R mutants. However, amino-acid substitution K42R leaves the salt bridge to H27 intact (Fig. 3C). Thus, the general structure of the A-site remains intact and rate and fidelity of translation remains unaffected [17]. This is in agreement with the observation that K42R is the only known mutation in S12 that confers streptomycin resistance but at the same time does not result in a hyper-accurate (i.e. restrictive) phenotype [3].

In contrast to K42R, mutations K42A or K42T disrupt the salt bridge to H27 (Fig. 3D, 3E). This mostly accounts for the restrictive phenotype of these mutations [17]. While K42A does not interfere with the binding of paromomycin directly [5], the hyperaccurate phenotype of the variant K42A ribosome in part functionally antagonizes aminoglycoside-induced misreading. Thus, these ribosomes show paromomycin-induced misreading only at much higher drug concentrations compared to wild-type [5].

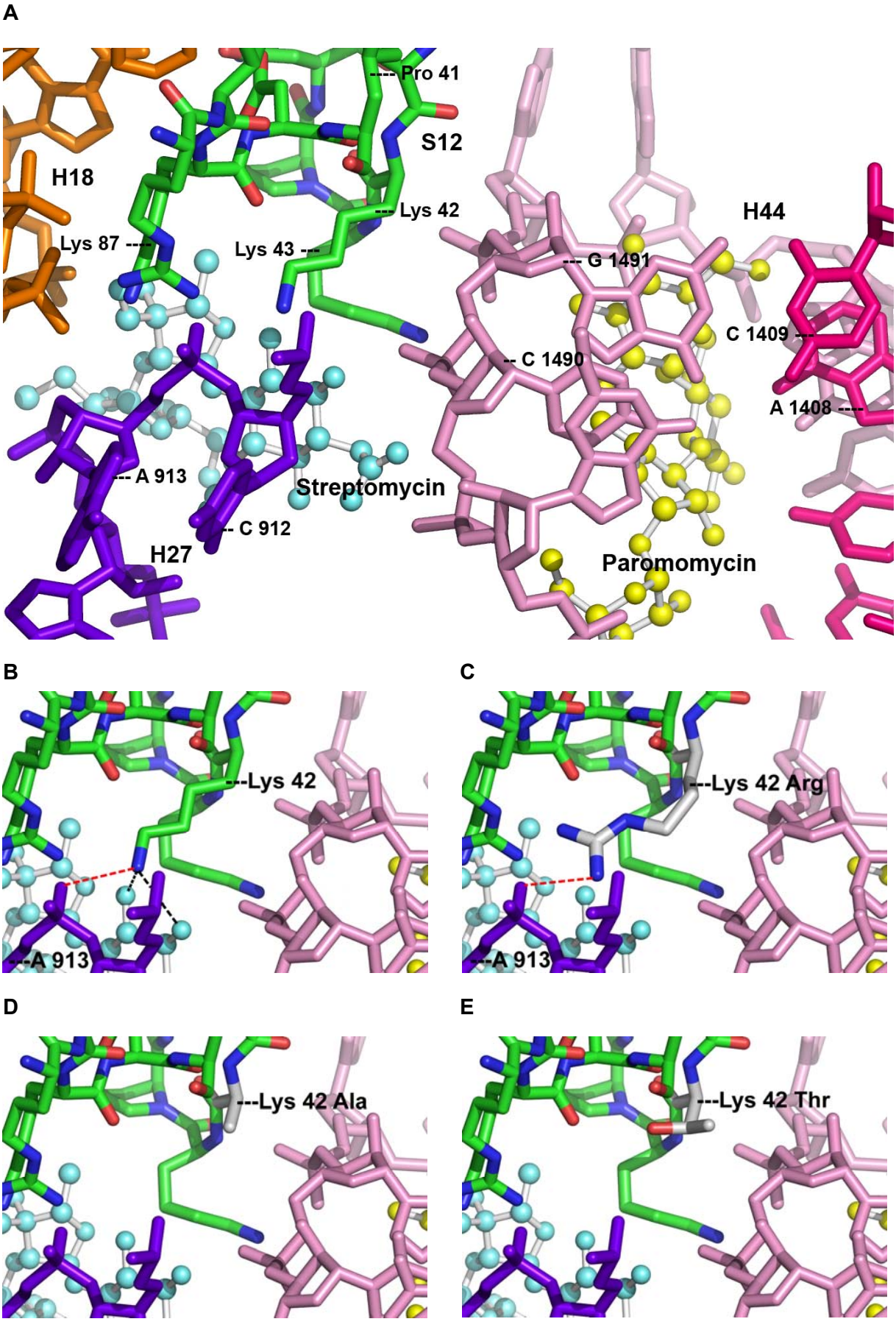
A
---- Pro 41
S12
H18
--- Lys 42
Lys 87 ----
Lys 43 ---
H44
--- G 1491
C 1409 ---
-- C 1490
A 1408 ----
--- A 913
Streptomycin
-- C 912
H27
Paromomycin
B
---Lys 42
---A 913
C
---Lys 42 Arg
---A 913
D
---Lys 42 Ala
E
---Lys 42 Thr

Figure 3. Three-dimensional crystal structure of *T. thermophilus* ribosomal decoding A-site with bound paromomycin and streptomycin. (**A**) General view of the A-site. Amino-acid residues of S12 (green) are shown labelled according to atoms: carbon – green, nitrogen – blue, oxygen – red. 16S rRNA helices are indicated as follows: H44 strand I (pink), H44 strand II (magenta), H27 (violet), H18 (orange). Streptomycin (light blue) and paromomycin (yellow). (**B**) Close up of wild type K42. Hydrogen bonds to streptomycin (black dotted lines) and salt bridge to A913 (red dotted line) are shown. (**C**) Close up of mutant K42R. Salt bridge (red dotted line) is shown. (**D**) Close up of mutant K42A (**E**) Close up of mutant K42T. (Protein Data Bank, 1FJG.pdb)

Conclusions

Our data demonstrate that K42R in ribosomal protein S12 does not affect resistance to 2-deoxystreptamine aminoglycosides as conferred by 16S-rRNA mutations in H44, and that S12 most likely plays little role in the species-specific pattern of susceptibility to 2-deoxystreptamines. This conclusion is supported by the observation that mutations G1645A/A1754G in *S. cerevisiae* 18S rRNA (homologous to *E. coli* residue A1408/G1491) reversed the natural resistance of yeast cytoplasmic ribosomes, i.e, increased the susceptibility to aminoglycosides from completely resistant (MIC > 5000 µg/ml) up to highly susceptible (MIC about 3 µg/ml). These levels of drug susceptibility are similar to those found in drug susceptible *E. coli* (MIC about 2.5-5 µg/ml) [20]. Thus, irrespective of ribosomal protein S12 (*S. cerevisiae* or *E. coli*), susceptibility to 2-deoxystreptamines is apparently determined by the nucleotide residues in small-subunit rRNA's drug binding pocket. This finding contrasts with the view of a universal interrelation between S12, base pair 1409-1491 and 2-deoxystreptamine susceptibility. Instead, it suggests that functional interactions between S12 and the 2-deoxystreptamine binding site are limited to certain bacterial genera and/or specific *rpsL* mutations. Interaction of S12 and 2-deoxystreptamine aminoglycosides apparently is not at the level of drug binding, but provoked by interference of two opposite effects on translation fidelity induced by error-restrictive *rpsL* mutations and misreading-inducing aminoglycoside antibiotics. As a result, this interplay is limited to S12 amino acid substitutions which confer a hyperaccurate phenotype and thus are functional antagonists of drug-induced misreading.

Materials and Methods

Strains used in this study

A single rRNA allelic strain *M. smegmatis* Δ*rrnB* (SZ380) was generated by unmarked deletion mutagenesis of the *rrnB* operon in *M. smegmatis* mc^2155. A suicide vector pH022 containing two DNA fragments flanking the *rrnB* operon (generated by PCR), a selectable marker (Gm^R) and counter-selectable marker *sacB* (both outside the *rrnB* DNA fragments), was transformed into *M. smegmatis* mc^2155. A single rRNA allelic derivative was obtained by

a two-step selection procedure: selection of transformants on agar plates containing gentamicin followed by a counter-selection step on agar plates with sucrose. Deletion of *rrnB* was confirmed by Southern blot analysis and partial sequencing.

Mutagenesis of the H44 decoding-site RNA was performed in strain *M. smegmatis* Δ*rrnB* (SZ0380) and in strain *M. smegmatis* Δ*rrnB rpsL* K42R (SZ0004) [12]. rRNA mutations were generated by PCR, cloned into vector pMIH-rrnB and introduced into the 16S rRNA A-site of the single rRNA allelic strain SZ0380 by RecA-mediated homologous recombination as described [12]. For a list of strains and plasmids see Tables S1 and S2 in supplementary data.

Minimal inhibitory concentration (MIC) assay

Drug susceptibility was studied by determining Minimal Inhibitory Concentrations (MIC). MIC tests were performed in a microtiter plate format as described [12]. In brief, freshly grown *M. smegmatis* cultures were resuspended in LB broth supplemented with 0.05% of Tween 80, diluted to an absorbance at 600 nm of 0.025 and incubated in the presence of 2-fold serial dilutions of 2-deoxystreptamine aminoglycosides. After incubation at 37°C for 72 h, the MIC was recorded as the lowest concentration of drug inhibiting visible growth.

Structural modelling

PyMol (DeLano Scientific) was used to render the structure of the A-site of 30S ribosomal subunit from *T. thermophilus* (Protein Data Bank, 1FJG.pdb) [21]

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22. Hobbie SN, Kalapala SK, Akshay S, Bruell C, Schmidt S, et al. (2007) Engineering the rRNA decoding site of eukaryotic cytosolic ribosomes in bacteria. Nucleic Acids Res 35: 6086-6093.

Supplementary data

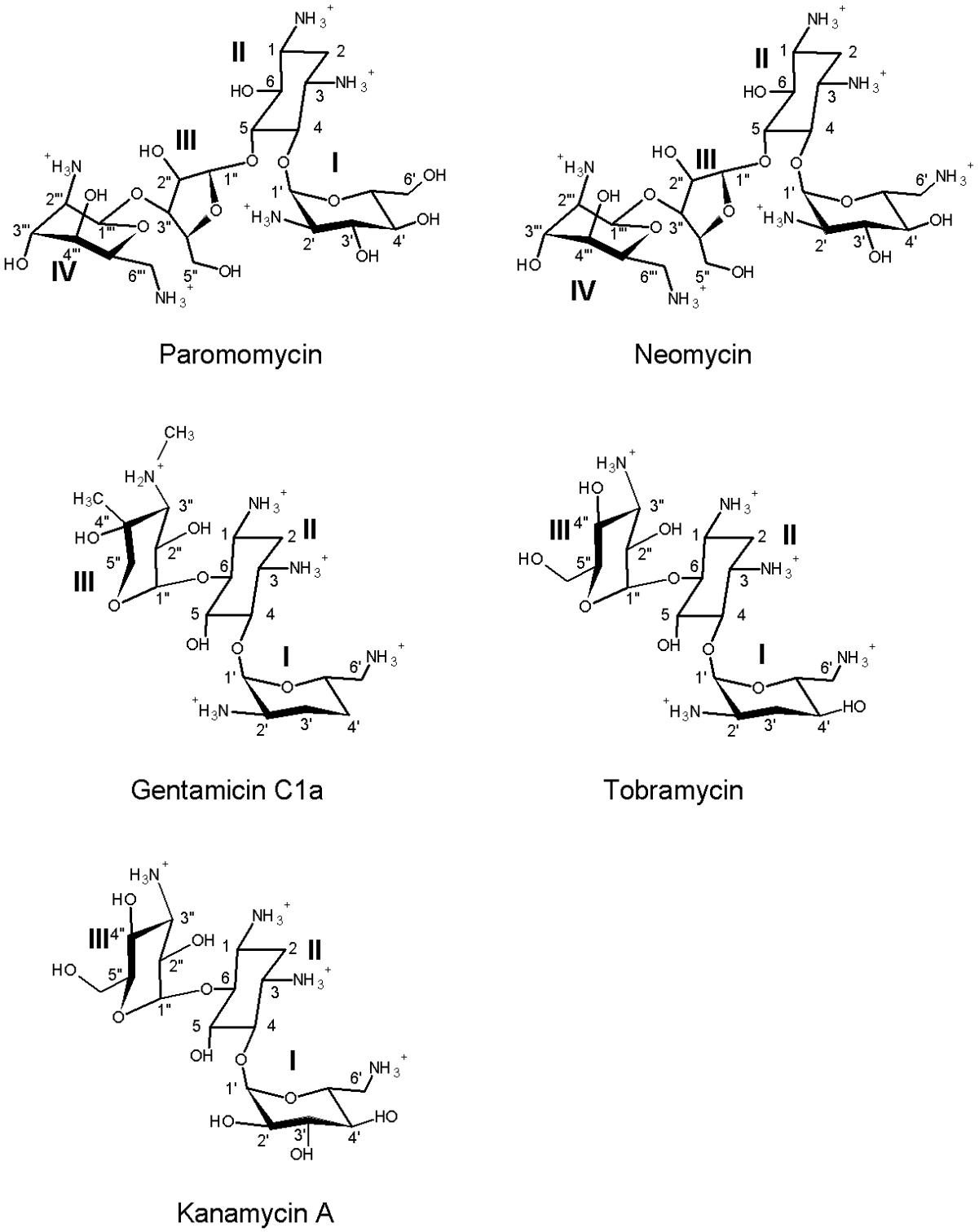

Figure S1. Chemical structures of disubstituted 2-deoxystreptamine antibiotics used in this study.

Table S1. Strains used in this study

M. smegmatis strain	Parental Strain	*rpsL*	16S rRNA mutation	Mutagenesis	Reference
SZ0380 Δ*rrnB rrnA*$^+$	---	wt	wt	---	[22]
SZ0004 Δ*rrnB rrnA*$^+$	---	K42R	wt	Spont.	[12]
Δ*rrnB rrnA*(G1491A)	SZ0380	wt	G1491A	Recomb.	this study
Δ*rrnB rrnA*(G1491A)	SZ0004	K42R	G1491A	Recomb.	[12]
Δ*rrnB rrnA*(G1491C)	SZ0380	wt	G1491C	Recomb.	this study
Δ*rrnB rrnA*(G1491C)	SZ0004	K42R	G1491C	Recomb.	[12]
Δ*rrnB rrnA*(G1491U)	SZ0380	wt	G1491U	Recomb.	this study
Δ*rrnB rrnA*(G1491U)	SZ0004	K42R	G1491U	Recomb.	[12]
Δ*rrnB rrnA*(C1409G)	SZ0380	wt	C1409G	Recomb.	this study
Δ*rrnB rrnA*(C1409G)	SZ0004	K42R	C1409G	Recomb.	[12]
Δ*rrnB rrnA*(C1409U)	SZ0380	wt	C1409U	Recomb.	this study
Δ*rrnB rrnA*(C1409U)	SZ0004	K42R	C1409U	Recomb.	[12]
Δ*rrnB rrnA*(A1408G)	SZ0380	wt	A1408G	Recomb.	this study
Δ*rrnB rrnA*(A1408G)	SZ0004	K42R	A1408G	Recomb.	[12]

Spont., spontaneous point mutation; Recomb., mutagenesis by RecA-mediated gene conversion.

Table S2. Plasmids used in this study

Number	Plasmid	*Marker*	16S rRNA mutations	Reference
pH022	pGEM7-*rrnB5'3'-sacB-aph*	Gm	-	this study
PZ176	pMV361Δ*aph-hyg*-1491A	Hyg	G1491A	[12]
PZ178	pMV361Δ*aph-hyg*-1491C	Hyg	G1491C	[12]
PZ177	pMV361Δ*aph-hyg*-1491U	Hyg	G1491U	[12]
PZ191	pMV361Δ*aph-hyg*-1409G	Hyg	C1409G	[12]
PZ175	pMV361Δ*aph-hyg*-1409U	Hyg	C1409U	[12]
pH128	pMV361Δ*aph-hyg*-1408G	Hyg	A1408G	[13]

Hyg, hygromycin; Gm, gentamicin.

Personal contributions

My personal contributions to the projects described in the results section were as follows

Project 1 - Engineering the rRNA decoding site of eukaryotic cytosolic ribosomes in bacteria. (2007). *Nucleic Acids Res*. 35(18):6086-93.

Hobbie, S. N.*, **Kalapala, S. K.*,** Akshay, S.*, Bruell, C. M., Schmidt, S., Dabow, S., Vasella, A., Sander, P., Böttger, E. C.

* Equal contribution

- Prepared ribosomes for *in-vitro* studies
- Generated aminoglycoside-induced inhibition of AUG$(UUU)_{12}$-driven phenylalanine incorporation data to calculate the IC_{50} values
- Developed TnT assay for mycobacterial ribosomes
- Generated aminoglycoside-induced inhibition of luciferase synthesis data by TnT assay to calculate the IC_{50} values

Project 2 – Genetic analysis of interactions with eukaryotic rRNA identify the mitoribosome as target in aminoglycoside ototoxicity. (2008). *Proc Natl Acad Sci U S A*. 105(52):20888-93.

Hobbie, S. N.*, Akshay, S.*, **Kalapala, S. K.*,** Bruell, C. M., Shcherbakov, D., Böttger, E. C.

* Equal contribution

- Prepared ribosomes for *in-vitro* studies
- Generated aminoglycoside-induced inhibition of AUG$(UUU)_{12}$-driven phenylalanine incorporation data to calculate the IC_{50} values
- Generated aminoglycoside-induced inhibition of luciferase synthesis data by TnT assay to calculate the IC_{50} values
- Participated in manuscript writing

Project 3 – Mutation K42R in ribosomal protein S12 does not affect susceptibility of *Mycobacterium smegmatis* 16S rRNA A- site mutants to 2-deoxystreptamine aminoglycosides. *(manuscript in preparation)*

Kalapala, S. K., Hobbie, S. N., Böttger, E. C., Shcherbakov, D.

- Constructed recombinant mutant strains
- Generated MIC data
- Wrote manuscript together with Shcherbakov, D., Hobbie, S. N., and Böttger, E. C.

3. Publications

1. **Kalapala, S. K.,** Hobbie, S. N., Böttger, E. C., Shcherbakov, D. Mutation K42R in ribosomal protein S12 does not affect susceptibility of *Mycobacterium smegmatis* 16S rRNA A-site mutants to 2-deoxystreptamine aminoglycosides. (manuscript in preparation)
2. Hobbie, S. N.*, Akshay, S.*, **Kalapala, S. K.*,** Bruell, C. M., Shcherbakov, D., Böttger, E. C. (2008). Genetic analysis of interactions with eukaryotic rRNA identify the mitoribosome as target in aminoglycoside ototoxicity. *Proc Natl Acad Sci U S A*. 105(52):20888-93.
3. Pathak, R., Perez-Fernandez, D., Nandurdikar, R., **Kalapala, S.K.,** Böttger, E.C., Vasella, A. (2008). Synthesis and Evaluation of Paromomycin Derivatives Modified at C(4'). *Helv. Chim. Acta. 91,* 1533-1552*.*
4. Hobbie, S. N.*, Bruell, C. M.*, Akshay, S., **Kalapala, S. K**., Shcherbakov, D., Böttger, E. C. (2008). Mitochondrial deafness alleles confer misreading of the genetic code. *Proc Natl Acad Sci U S A*. 105(9):3244-49.
5. Hobbie, S. N.*, **Kalapala, S. K.*,** Akshay, S.*, Bruell, C. M., Schmidt, S., Dabow, S., Vasella, A., Sander, P., Böttger, E. C. (2007). Engineering the rRNA decoding site of eukaryotic cytosolic ribosomes in bacteria. *Nucleic Acids Res*. 35(18):6086-93.
6. Hobbie, S. N., Bruell, C. M., **Kalapala, S**., Akshay, S., Schmidt, S., Pfister, P., Böttger, E. C. (2006). A genetic model to investigate drug-target interactions at the ribosomal decoding site. *Biochimie* 88(8):1033-43.

* These authors contributed equally to this work

4. Conference presentations

2009 EMBO Conference on Protein Synthesis and Translational Control, Heidelberg, Germany
rRNA sequence polymorphism within the bacterial domain and susceptibility to drugs targeting protein synthesis
R. Akbergenov, A. Subramanian, **S. K. Kalapala**, M. Bertea, M.K.A. Kulstrunk and E.C. Böttger

EMBO Conference on Ribosome Synthesis, Regensburg, Germany
Probing of aminoglycoside interaction with the ribosomal decoding A-site by site-directed mutagenesis
S.K. Kalapala, S. Akshay, D. Scherbakov, R. Akbergenov and E.C. Böttger

12S rRNA deafness alleles and aminoglycoside induced ototoxicity converge on mitochondrial mistranslation as mechanism of disease pathogenesis
S. Akshay, **S.K. Kalapala**, R. Akbergenov, D. Shcherbakov and E.C. Böttger

68th Annual Assembly of the Swiss Society for Microbiology (SSM), Lausanne, Switzerland
Synthesis of novel aminoglycoside compounds by a synthetic biology approach
E.C. Böttger, D.P. Fernandez, I. Kudyba, R. Pathak, S. Harish, S.R. Dubakka, **S.K. Kalapala**, D. Scherbakov, R. Akbergenov and A. Vasella

Aminoglycosides: Structural analyses of interactions with eukaryotic A-sites point to the mitoribosome as target and identify mechanisms of toxicity
D. Scherbakov, S. Akshay, **S.K. Kalapala**, M.K.A. Kulstrunk, R. Akbergenov and E.C. Böttger

2008 60th Annual Meeting of the German Society for Microbiology (DGHM), Dresden, Germany
Synthesis of novel aminoglycoside compounds by a synthetic biology approach
E.C. Böttger, D.P. Fernandez, I. Kudyba, R. Pathak, S. Harish, S.R. Dubakka, **S.K. Kalapala**, S.N. Hobbie and A. Vasella

Aminoglycosides: Structural analyses of interactions with eukaryotic A-sites point to the mitoribosome as target and identify mechanisms of toxicity
S.N. Hobbie, S. Akshay, **S.K. Kalapala**, C.M. Bruell, D. Scherbakov and E.C. Böttger

13th Annual Meeting of the RNA Society, Berlin, Germany
Mutations A1555G and C1494U in mitochondrial 12S rRNA affect the accuracy of mRNA translation
S. Metze, **S.K. Kalapala**, S.N. Hobbie and E.C. Böttger

IUBMB Symposium on Mitochondrial Physiology and Pathology, Bari, Italy
Mitochondrial deafness alleles affect the accuracy of mRNA translation
S.N. Hobbie, S. Akshay, **S.K. Kalapala**, C.M. Bruell, D. Scherbakov and E.C. Böttger

EMBO Conference on RNA and Disesse, Rome, Italy
Mitochondrial 12S rRNA mutations increase ribosomal susceptibility to aminoglycoside-induced mistranslation
D. Scherbakov, S.N. Hobbie, **S.K. Kalapala**, S. Akshay and E.C. Böttger

67th Annual Assembly of the Swiss Society for Microbiology (SSM) 2008, Interlaken, Switzerland
Transplanting eukaryotic decoding sites into bacterial hybrid ribosomes
S.K. Kalapala, S. Akshay, C.M. Bruell, S.N. Hobbie and E.C. Böttger

2007 EMBO Conference on Protein Synthesis and Translational Control, Heidelberg, Germany
Genetic modeling of Mycobacterium smegmatis ribosomal RNA
S.K. Kalapala, S.N. Hobbie and E.C. Böttger

Transplanting human and protozoan decoding sites into bacterial hybrid ribosomes
S.N. Hobbie, **S.K. Kalapala**, S. Akshay, C.M. Bruell and E.C. Böttger

4th International Conference on the Ribosome, Cape Cod, USA
Modeling the rRNA decoding site of eukaryotic cytosolic ribosomes in bacteria
S.N. Hobbie, C.M. Bruell, **S. K. Kalapala**, S. Akshay and E.C. Böttger

2006 2nd FEMS Congress of European Microbiologists, Madrid, Spain
Genetic analysis of aminoglycoside binding to the ribosomal decoding site
S.N. Hobbie, **S.K. Kalapala**, A. Subramanian, B. François, E. Westhof and E.C. Böttger

CURRICULUM VITAE

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EDUCATION AND RESEARCH ACTIVITIES

2005 – Present:

Doctoral studies under the supervision of Prof. Dr. Erik C. Böttger, Institute of Medical Microbiology, University of Zurich, Zurich, Switzerland.

"Study of Aminoglycoside Antibiotics Interaction with Eukaryotic Ribosomal Decoding Sites Using Bacterial Hybrid Ribosomes"

2001 – 2004:

I had worked as a **Research assistant** under the supervision of Prof. Umesh Varshney, Department of Microbiology and Cell Biology, Indian Institute of Science, Bangalore, India

"Studies on ribosome recycling factor from mycobacteria"

1999 – 2001:

Graduate studies in Microbiology, Nagarjuna University, Guntur, India
Graduate Thesis under the supervision of Dr. Ashlesha Deshpande, Indian Institute of Chemical Technology, Hyderabad, India.

"Enzyme immobilization / purification: a novel approach on protease, an extra cellular enzyme produced by Bacillus licheniformis".

1996 – 1999:

Undergraduate studies in Microbiology, Biochemistry, Chemistry and Aqua Culture, Nagarjuna University, Guntur, India.

1993 – 1996:

Intermediate education in Botany, Zoology, Physics and Chemistry, SRR & CVR Govt. College, Vijayawada, India.

1983 – 1993:

Primary and Secondary school education at Tagore, Jaikisan and Gowtham public schools, Vijayawada, India.

LIST OF PUBLICATIONS

2010 **Kalapala, S. K.,** Hobbie, S. N., Böttger, E. C., Shcherbakov, D. Mutation K42R in ribosomal protein S12 does not affect susceptibility of *Mycobacterium smegmatis* 16S rRNA A-site mutants to 2-deoxystreptamine aminoglycosides. (manuscript in preparation)

2008 Hobbie, S. N.*, Akshay, S.*, **Kalapala, S. K.*,** Bruell, C. M., Shcherbakov, D., Böttger, E. C. (2008). Genetic analysis of interactions with eukaryotic rRNA identify the mitoribosome as target in aminoglycoside ototoxicity. *Proc Natl Acad Sci U S A*. 105(52):20888-93.

2008 Pathak, R., Perez-Fernandez, D., Nandurdikar, R., **Kalapala, S.K.,** Böttger, E.C., Vasella, A. (2008). Synthesis and Evaluation of Paromomycin Derivatives Modified at C(4'). *Helv. Chim. Acta. 91,* 1533-1552*.*

2008 Hobbie, S. N.*, Bruell, C. M.*, Akshay, S., **Kalapala, S. K**., Shcherbakov, D., Böttger, E. C. (2008). Mitochondrial deafness alleles confer misreading of the genetic code. *Proc Natl Acad Sci U S A*. 105(9):3244-49.

2007 Hobbie, S. N.*, **Kalapala, S. K.*,** Akshay, S.*, Bruell, C. M., Schmidt, S., Dabow, S., Vasella, A., Sander, P., Böttger, E. C. (2007). Engineering the rRNA decoding site of eukaryotic cytosolic ribosomes in bacteria. *Nucleic Acids Res*. 35(18):6086-93.

2006 Hobbie, S. N., Bruell, C. M., **Kalapala, S**., Akshay, S., Schmidt, S., Pfister, P., Böttger, E. C. (2006). A genetic model to investigate drug-target interactions at the ribosomal decoding site. *Biochimie* 88(8):1033-43.

2005 Saikrishnan, K., **Kalapala, S. K**., Varshney, U., Vijayan, M. (2005) X-ray structural studies of Mycobacterium tuberculosis RRF and a comparative study of RRFs of known structure. Molecular plasticity and biological implications. *J Mol Biol.*345(1):29-38.

2004 Saikrishnan, K., **Kalapala, S. K**., Bidya Sagar, M., Rao, A.R., Varshney, U., Vijayan, M. (2004) Purification, crystallization and preliminary X-ray studies of Mycobacterium tuberculosis RRF. *JActa Crystallogr. D Biol. Crystallogr.* 60(Pt2):368-70.

* These authors contributed equally to this work